IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Jun-Ichi Nezu *et al.*	Art Unit	: 1647
Serial No.	: 10/762,154	Examiner	: Bridget E. Bunner
Filed	: January 21, 2004	Conf. No.	: 4898
Title	: POLYNUCLEOTIDES ENCODING hOCTN1 POLYPEPTIDE		

Mail Stop Appeal Brief - Patents
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BRIEF ON APPEAL

Appellant is appealing the final rejection of claims 8, 10, 11, 13, 16, 18-21, 23-25, 27, 32 and 36 in the Final Office Action dated September 27, 2007, as modified and elaborated in the Advisory Actions respectively dated April 11, 2008, and August 1, 2008. An Amendment after Final was filed on February 25, 2008, and received by the U.S. Patent and Trademark Office on that date. A Notice of Appeal was filed on March 26, 2008, and received by the U.S. Patent and Trademark Office on that date.

(i) Real Party in Interest

The Real Party in Interest is Chugai Seiyaku Kabushiki Kaisha, the assignee of record.

(ii) Related Appeals and Interferences

There are no prior or pending related appeals, judicial proceedings, or interferences.

(iii) Status of Claims

Claims 1-7, 9, 12, 14, 15, 17, 22, 26 and 28-31 are canceled.

Claims 33-35 are withdrawn.

Claims 8, 10, 11, 13, 16, 18-21, 23-25, 27, 32 and 36 are rejected and under appeal.

(iv) Status of Amendments

The Advisory Action dated August 1, 2008 (henceforth "the Advisory Action") states that, for purposes of appeal, all previously filed amendments have been entered. No amendments are being submitted herewith.

(v) **Summary of Claimed Subject Matter**

The claims under appeal are directed to (a) isolated nucleic acids encoding an organic cation transporter protein, hOCTN1, and certain variants thereof; (b) vectors and cultured host cells containing the isolated nucleic acids; and (c) methods of producing the encoded polypeptides. Withdrawn method claims 33-35, directed to methods of screening compounds for the ability to be transported by the polypeptide encoded by the nucleic acid of claim 8, are not under appeal; however, they are retained in the application for possible rejoinder, in accordance with MPEP § 821.04, if claim 8 is deemed allowable. Claims 8, 10, 11 and 36 are the independent claims under appeal. As all of the claims are individually addressed in the arguments below, all are summarized here.

Claim 8 is directed to an isolated nucleic acid encoding a polypeptide that includes the sequence of SEQ ID NO:1. SEQ ID NO:1 is the amino acid sequence of a human protein, hOCTN1, discovered by the inventors. Support for independent claim 8 can be found in original claims 5, 8, and 9, as well as in the specification, *e.g.*, at page 6, lines 6-23.

Claim 10 is directed to an isolated nucleic acid encoding a polypeptide that contains the amino acid sequence of SEQ ID NO:1 with one to 30 conservative amino acid substitutions, where the polypeptide is a transporter of an organic cation. Support for independent claim 10 can be found in original claims 10 and 6, as well as in the specification on page 5, lines 12-21, and page 9, lines 1-14.

Claim 11 is directed to an isolated nucleic acid that hybridizes under stringent conditions to a probe, where the sequence of the probe consists of the complement of SEQ ID NO:2, and where the isolated nucleic acid encodes a polypeptide that is a transporter of an organic cation. The stringent conditions include hybridization at 68 °C followed by washing in 2 X SSC/0.1% SDS for 20 minutes at room temperature and twice in 0.1 X SSC/0.1% SDS for 20 minutes at 50 °C. SEQ ID NO:2 is the sequence of cDNA encoding the hOCTN1 protein of SEQ ID NO:1. Support for independent claim 11 can be found in original claim 11, as well as in the specification, *e.g.,* at page 6, lines 3-5, at page 10, lines 13-31, and at page 11, line 21, to page 12, line 14.

Claim 13 is directed to the nucleic acid of claim 11, wherein the amino acid sequence of the polypeptide comprises SEQ ID NO:1. Claim 11 and its support are summarized above. Claim 13 is supported by original claims 11, 12 and 13, and by the specification, *e.g.,* at page 6, lines 3-11.

Claim 16 is directed to a vector comprising the nucleic acid of claim 8. Claim 8 and its support are described above. Claim 16 is an original claim, so provides its own written description. It is also supported in the specification at, *e.g.,* page 5, line 23, and in Example 6.

Claim 18 is directed to a vector comprising the nucleic acid of claim 10. Claim 10 and its support are described above. Claim 18 is an original claim, so provides its own written description. It is also supported in the specification at, *e.g.,* page 5, line 23.

Claim 19 is directed to a vector comprising the nucleic acid of claim 11. Claim 11 and its support are described above. Claim 19 is an original claim, so provides its own written description. It is also supported in the specification at, *e.g.,* page 5, line 23.

Claim 20 is directed to a vector comprising the nucleic acid of claim 13. Claim 13, in turn, depends from claim 11, which is described above. Claim 13 further limits the nucleic acid of claim 11 to a nucleic acid encoding a polypeptide with an amino acid sequence that includes SEQ ID NO:1. Claim 20 is supported by original claims 11-13 and 20. It is also supported in the specification at, *e.g.,* page 5, line 23.

Claim 21 is directed to a cultured host cell comprising the nucleic acid of claim 8. Claim 8 and its support are described above. Claim 21 is an original claim, so provides its own written description. It is also supported in the specification at, *e.g.,* page 5, lines 24-28, and in Example 6.

Claim 23 is directed to a cultured host cell comprising the nucleic acid of claim 10. Claim 10 and its support are described above. Claim 23 is an original claim, so provides its own written description. It is also supported in the specification at, *e.g.,* page 5, lines 24-28.

Claim 24 is directed to a cultured host cell comprising the nucleic acid of claim 11. Claim 11 and its support are described above. Claim 24 is an original claim, so provides its own written description. It is also supported in the specification at, *e.g.,* page 5, lines 24-28.

Claim 25 is directed to a cultured host cell comprising the nucleic acid of claim 13. Claim 13, in turn, depends from claim 11, which is described above. Claim 25 is supported by original claims 11, 12, and 25, as well as page 5, lines 17-28, and Example 6.

Claim 27 is directed to a method of producing a polypeptide, the method comprising isolating the polypeptide from the cultured host cell of claim 21. Claim 21, in turn, is drawn to a cultured host cell comprising the nucleic acid of claim 8. Claims 8 and 21 are summarized above. Claims 27 and 21 are both originally filed claims, so provide their own support. They find further support in the specification, *e.g.,* at page 5, lines 26-28; page 7, lines 14-16; and page 12, line 20, to page 13, line 14.

Claim 32 is directed to the nucleic acid of claim 10, further specifying that the sequence of the encoded polypeptide comprises SEQ ID NO:1 with up to 10 conservative amino acid substitutions. Claim 10 and its support are described above. Claim 32 is supported in the specification, *e.g.,* at page 10, lines 4-6.

Claim 36 is directed to an isolated nucleic acid encoding a polypeptide that consists of the sequence of SEQ ID NO:1. Support for claim 36 can be found in the specification, *e.g.*, at page 6, lines 6-11.

(vi) Grounds of Rejection to be Reviewed on Appeal

Claims 8, 10, 11, 13, 16, 18-21, 23-25, 27, 32 and 36 are rejected under 35 U.S.C. §101 as allegedly lacking utility.

Claims 8, 10, 11, 13, 16, 18-21, 23-25, 27, 32 and 36 are rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the enablement requirement.

Claims 10, 18, 23 and 32 are rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement.

(vii) Argument

I. Rejection on Grounds of Lack of Utility under 35 U.S.C. §101

Claims 8, 10, 11, 13, 16, 18-21, 23-25, 27, 32 and 36 are rejected under 35 U.S.C. §101 as allegedly lacking a well-established utility or a credible, substantial and specific asserted utility. The Examiner acknowledges that the claimed nucleic acids and their encoded hOCTN1

transporter polypeptides can be used to screen for and transport carcinostatic compounds, but asserts that these do not qualify as specific and substantial uses because, like the compounds and nucleic acids respectively at issue in the Brenner (*Brenner v. Manson*, 148 U.S.P.Q. 689 (Sup. Ct. 1966)) and Fisher (*In re Fisher*, 421 F.3d 1365, 76 USPQ2d 1225 (Fed. Cir. 2005)) cases, "further research is required to identify or reasonably confirm a specific and substantial utility." According to the Advisory Action, "Although the hOCTN1 protein of the instant application is able to transport carcinostatics, the physiological function of the protein has yet to be determined."

A. The Asserted Utility is Specific, Substantial and Credible

Appellant pointed out in the Amendment after Final that the specification discloses use of the hOCTN1 transporter protein encoded by the presently claimed nucleic acids in a screen for carcinostatic compounds transported by the protein. The Advisory Action acknowledges that the specification discloses such a use, but maintains that **"screening for carcinostatics is not specific or substantial. Such assays can be performed with any polypeptide. The specification discloses nothing specific or substantial for the compounds screened in this method."** The Examiner's position is not understood.

First, Appellants point out the rather obvious fact that, by definition, carcinostatic compounds that are identified in a screen with hOCTN1 transporter protein have the specific and substantial use of being carcinostatic compounds (i.e., able to inhibit growth of cancerous cells). It is not clear why the Examiner believes otherwise.

Second, Appellants are mystified as to why the Examiner asserts that **"Such assays can be performed with any polypeptide."** No explanation is provided as to why anyone would wish to perform such an assay with "any" polypeptide, including those that do not possess transporter activity. Perhaps it is theoretically possible to attempt to perform *any* assay with *any* polypeptide, but obviously most of such attempts with random polypeptides not previously identified as having a relevant activity would be doomed to failure. Appellant is not proposing some random screening assay that is no more applicable to the presently claimed nucleic acids than to any others, and thus lacks specificity. Rather, Appellant's asserted utility is narrow and specific to the *particular* activity identified by Appellant for the presently claimed nucleic acids:

transport of organic cations, including carcinostatic compounds. Appellant has demonstrated that hOCTN1 is able to transport organic cations, including some that are carcinostatic compounds. See, e.g., Examples 6 and 7. The Examiner does not dispute that hOCTN1 possesses this activity. Appellant has asserted that, because of this demonstrated activity, the presently claimed nucleic acids, vectors, and cells are useful in screening assays designed to develop new drugs that can be transported by hOCTN1 (see, e.g., page 31, lines 5-16, and page 35, lines 6-12). That a screen for anti-cancer drugs qualifies as a "substantial" utility is without serious question. That a screen for anti-cancer drugs that are transported by an organic cation transporter protein qualifies as a "specific" utility is also clear. These points are discussed in detail below.[1]

Specific Utility

The Office has released "Guidelines for Examination of Applications for Compliance with the Utility Requirement," which address utility under 35 U.S.C. §101 and 35 U.S.C. §112, first paragraph ("Utility Guidelines"), and an "Overview of Legal Precedent Governing the Utility Requirement" ("Legal Overview") to support the Utility Guidelines. The Utility Guidelines define a "specific" utility as one that is particular to the subject matter claimed (just as in the present situation).

The Utility Guidelines provide an example of a nucleic acid with a "non-specific" utility as one where the asserted utility is that of a "gene probe," without specifying the gene target to which it can bind. This would be comparable to the present facts only if Appellant's specification has failed to say anything about hOCTN1 other than that it could be used in generic "drug screening", with no clue as to what sort of drug it would be capable of finding, or for what type of disease, or how the screen would be carried out.

This is far from being the case with regard to the instant claims. The specification clearly and extensively characterizes the structural features and biological activity of the newly discovered OCTN1 gene family. The teachings of the specification establish that this new gene

[1] The Examiner has not challenged the credibility of the asserted utility, so that prong of the utility requirement is taken as established and will not be discussed here.

family belongs to the general class of organic cation transporter proteins, several of which were known and previously studied in biological systems. Based on the teachings as a whole, the specification asserts a utility for the claimed isolated nucleic acids, namely, expressing the encoded hOCTN1 transporter proteins in cells and screening for carcinostatics that are preferentially absorbed and transported by hOCTN1. The screen permits the selection of carcinostatics that will preferentially be absorbed by target tissues or cells that express the hOCTN1 transporter. If one is trying to treat a cancer in which the cancerous cells express the hOCTN1 transporter, one can use the screen to determine whether a given known carcinostatic compound would be effectively taken up by those cells. Similarly, the screen can be used to identify new carcinostatic compounds that are preferentially absorbed and transported by hOCTN1, so are potentially useful for the same purpose. The specification asserts utility of the nucleic acids in expressing proteins that function as organic transporters of carcinostatic compounds, and illustrates this utility by showing that hOCTN1 actually transports known carcinostatins such as as actinomycin D, etoposide, vinblastine and daunomycin.

The asserted utility is therefore specific because it is particular to the type of protein, the hOCTN1 organic cation transporter, discovered by Appellant. Contrary to the Examiner's assertions, the screen is not broadly applicable to "any" protein. Rather, it is directly related to the disclosed function, that of an organic cation transporter, identified for the particular protein, hOCTN1, encoded by the claimed nucleic acids. Appellant has not merely hypothesized that the claimed nucleic acids and their encoded proteins "may be useful" in a general sense. See MPEP §2107.01. Rather, the specification teaches why these particular nucleic acids and their encoded proteins have specific uses, and then goes on to demonstrate the specific uses.

Substantial Utility

The MPEP, in discussing "substantial" utility at §2107.01, states that a "substantial utility" defines a "real world" use that does not require carrying out further research to identify or reasonably confirm the use. The Utility Guidelines provide an example of an insubstantial utility for a nucleic acid as one in which a claimed nucleic acid is used merely to study its own properties.

In this instance, the specification clearly meets the standard of providing a substantial utility. The specification teaches how the proteins encoded by the claimed nucleic acids can be used to study the transport of various organic cations, including carcinostatic agents, in cells, and can therefore be used to screen for compounds that are amenable to being transported by these proteins. The screening assays further find use in developing particular carcinostatic agents for treatment of different types of cancer, based on the expression of hOCTN1 in various tumor cell lines. No further research is necessary to confirm Appellant's identification and characterization of this gene as encoding organic cation transporter proteins, and indeed the Examiner does not challenge Appellant's characterization of hOCTN1 as an organic cation transporter. What is asserted as a utility is its use in assays to identify compounds (such as carcinostatic compounds) that are transported by hOCTN1, and so might be pharmacologically effective in certain diseases such as cancer. Thus, the asserted utility is substantial because it provides a significant, currently available, real-world benefit: an assay for finding the best cancer treatment for a cell or tissue that expresses the hOCTN1 transporter. The assay can select for the best treatment for a given patient's cancer from among several known carcinostatics, or can be used to find new carcinostatics. *There is no need to know anything more about the physiological role of hOCTN1 in order for the gene to be employed as disclosed.*

Distinguishing the *Brenner* and *Fisher* cases

The Examiner alleges in the Advisory Action that, unlike a scale or a microarray or a gas chromatograph, the claimed hOCTN1 nucleic acid molecules and their encoded polypeptides do not have patentable utility as "research tools" because "further research is required to identify or reasonably confirm a specific and substantial utility." The Examiner opines that the instant situation is analogous to the facts of *Brenner v. Manson,* 148 U.S.P.Q. 689 (Sup. Ct. 1966), because, like the chemical compounds in *Brenner*, the claimed nucleic acids encoding organic cation transporter proteins allegedly have utility only in the broadest sense and not in an immediately obvious or fully disclosed "real world" utility. The Examiner also analogizes the instant case to *In re Fisher*, 421 F.3d 1365, 76 USPQ2d 1225 (Fed. Cir. 2005), alleging that, like the nucleic acids in the *Fisher* case, the instantly claimed nucleic acids do not have a "significant and presently available benefit to the public."

Appellant believes that the Office has misapplied those cases to the present situation, as the facts of the present case differ from those in *Brenner* and *Fisher* in key ways. For example, the specification in *Brenner* failed to assert any utility--or even any potential activity--for the compound produced by the claimed process. The Court rejected Brenner's argument that, since the compound could be used in research to determine whether it possessed a useful activity, the claimed process met the utility requirement. In distinct contrast to the facts of *Brenner*, Appellant's specification not only describes in great detail an asserted utility for the presently claimed nucleic acids, but also demonstrates that this utility indeed works. Furthermore, the utility disclose in the instant specification is not merely use in experiments to discover a possible use. The citation of *Brenner* in support of the rejection is therefore inapposite.

In *Fisher*, the court held that claims drawn to five expressed sequence tags (ESTs) from the maize genome did not have a specific or substantial utility because they were useful only to find the underlying genes whose identity and usefulness had yet to be determined. Unlike the situation in *Fisher*, hOCTN1's utility is not limited to figuring out the identity and usefulness of the claimed nucleic acids (or underlying genes) themselves. The instantly claimed isolated nucleic acids encode a fully sequenced, structurally- and functionally-characterized protein, hOCTN1. The specification actually demonstrates the utility of hOCTN1 in transporting known carcinostatic organic cation compounds. The asserted utility is specific because it applies only to organic cation transporters, and is substantial because it is immediately applicable in a current, real-world sense: to select optimal carcinostatics for uptake by cancer cells/tissues that express the hOCTN1 transporter.

In light of the above, Appellant submits that the asserted utility is specific, substantial, and credible.

"Physiological Role" and Utility

Despite the above arguments, the Examiner continues to insist that satisfaction of the utility requirement in the present case would require more information about the "physiological role" of hOCTN1 than was provided in the specification. According to the Advisory Action, "**There is no biological activity, phenotype, disease or condition, binding partner, or any other specific feature that is disclosed as being associated with OCTN1.**" Appellant has

pointed out repeatedly that a biological activity (organic cation transport) is disclosed throughout the specification for OCTN1, and further that a useful screening assay that exploits that activity is also disclosed, and in fact *demonstrated* in the Examples. The Examiner dismisses this evidence of utility, apparently on the belief that the claimed nucleic acid would not be deemed useful by those of ordinary skill in the art unless the specification discloses what organic cations are naturally transported by the protein *in vivo* (i.e., the "physiological role" or "binding partner" of OCTN1), and/or some sort of differential expression in diseased cells versus normal cells to indicate an association of OCTN1 with a particular disease.[2] Appellant maintains that the Examiner has set a standard for the utility requirement that is nowhere in the law. Although the categories of information mentioned in the Advisory Action might well be useful in some situations to establish that a given nucleic acid meets the utility requirement, there is no basis in law to suppose that such information is absolutely required. Neither the statute nor the caselaw regarding the utility requirement, nor even the Utility Guidelines, sets forth such a standard. In the present case, Appellant has unequivocally demonstrated utility of the claimed nucleic acids, vectors and cells in an assay that is *specific* to organic cation transporters and is *substantial* in that it is a "real world" use. Nothing more should be required.

It may be helpful to analogize the present situation to a hypothetical discovery of a compound extracted from the bark of a tree and disclosed in the specification to be useful as a starting material for synthesis of related compounds having potent anticancer activity against tumors in animals. In this hypothetical fact pattern, nothing is known about the physiological role the compound performs in the tree, nor is there any link to a known disease of the tree. The asserted utility is as a starting material for synthesis of anticancer agents, a utility that may have nothing to do with its physiological role in the tree. There is no question that this hypothetical

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[2] During a telephonic interview with Appellant's undersigned representative on January 10, 2008, the Examiner attempted to justify her assertions regarding the utility requirement by saying that there were several recent unpublished decisions by the Board of Patent Appeals and Interferences (BPAI) that stood for this proposition. Because the Examiner was unable to name any of those decisions, Appellant reviewed several recent BPAI decisions that concerned rejections of newly discovered proteins or nucleic acids for lack of utility. The ones that appeared to Appellant to be most relevant to the present rejection were discussed in the Amendment after Final, where Appellant distinguished each from the present facts. In response, the Advisory Action explained that the rejection was not based on these non-precedential opinions of the Board, but rather on the statute. Appellant reiterates that neither the statute nor the *Brenner* and *Fisher* cases cited by the Examiner nor any of the non-precedential BPAI decisions that Appellant could locate supports the rejection of the present claims for lack of utility.

compound would be found to have patentable utility despite the lack of information about its physiological role. Similarly, the utility of the presently claimed nucleic acids and their encoded proteins disclosed in the specification does not hinge on knowledge of their "physiological role," nor on any association with a disease.

B. Well-established Utility

Appellant further submits that use of hOCTN1 in a screening assay is also a "well-established" utility, *i.e.*, even had it not been explicitly asserted in the specification, it nonetheless would have satisfied the utility requirement. This point was made in the Amendment after Final, but was not even addressed in the Advisory Action. As the references provided with the attached Appendix show, long before the instant application's earliest priority date, the distinct structural features that characterized organic cation transporter proteins[3] and their role in drug uptake and distribution in organs such as intestines,[4] kidneys,[5] and the liver[6] were well-known. Thus, regardless of what utilities are or are not asserted in the specification, the claimed isolated nucleic acids and vectors encode proteins that have a well-established utility, *i.e.*, one of ordinary skill in the art would immediately appreciate why the hOCTN1 protein is useful, based on disclosed structural and functional characteristics that establish its role as an organic cation transporter.

C. Claim-by-claim Analysis

Appellant understands the Examiner's rejection to apply equally to all of the pending claims, regardless of breadth and regardless of whether the claim is drawn to a nucleic acid, a vector, a host cell, or a method of producing a polypeptide. The arguments set forth above fully

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[3] See, Maiden *et al.*, *Nature*, 325:641-643 (1987), which was made of record in this case with the Amendment after Final and is included in the Evidence Appendix as Exhibit A.

[4] See, Tsuji *et al.*, *Pharm. Res.* 13(7):963-1132 (1996), which was made of record in this case with the Amendment after Final and is included in the Evidence Appendix as Exhibit B.

[5] See, Ullrich *et al.*, *Clin. Investig.* 71:843-848 (1993), which was made of record in this case with the Amendment after Final and is included in the Evidence Appendix as Exhibit C.

[6] See, Meijer *et al.*, *J. Pharmacokin. Biopharm.*, 18:35-70 (1990), which was made of record in this case with the Amendment after Final and is included in the Evidence Appendix as Exhibit D.

counter the rejection for lack of utility as formulated in the Office actions and Advisory Actions of record. If the Board chooses to maintain the rejection but formulate the rejection differently than did the Examiner (e.g., by finding only the broader claims, such as claims 10 and 11, to lack utility on some sort of grounds not applicable to the narrower claims, or *vice versa*), Appellant requests the opportunity to address that differently formulated rejection in re-opened prosecution.

Claims 8, 11, 13, and 36 are drawn to isolated nucleic acids. Each of these claims broadly encompasses SEQ ID NO:2, the cDNA sequence encoding SEQ ID NO:1 (hOCTN1). SEQ ID NO:2 could be used as a template to generate the complement of SEQ ID NO:2, which would possess the well-established utility of being useful as a probe to detect expression of hOCTN1 in a given patient's cancer cells, e.g., in Northern analysis as described in Examples 3 and 5. One could use the knowledge that hOCTN1 is or is not expressed in a patient's cancer cells to determine whether treatment with a particular organic cation carcinostatic compound would be worthwhile. If hOCTN1 is found to be expressed in the cancer cells, one would then select an organic cation carcinostatic compound that had been shown (in assays disclosed in the specification) to be transported by hOCTN1. Thus, these claims possess a utility in addition to the one discussed above that applies to all of the claims.

The nucleic acids of claims 8, 10, 11, 13, 32, and 36 and the vectors of claims 16, 18, and 19 are useful for producing the cultured host cells of claims 21, 23, 24, and 25. Those host cells can be used to express hOCTN1 polypeptide or a variant thereof, which in turn can be used to generate antibodies specific for hOCTN1 polypeptide (see, e.g., the specification at pages 7-8). The antibodies are useful to determine whether a given patient's cancer cells are expressing hOCTN1, and so can allow a physician to decide whether treatment with a particular organic cation carcinostatic compound known to be transported by hOCTN1 would be worthwhile (as described above).

Accordingly, there are multiple bases for finding that the present claims possess utility under 35 USC § 101. In view of the above, Appellant submits that the utility requirement is more than amply met in this case. Reversal of the rejection of all of the claims for lack of utility is respectfully requested.

II. Rejection for Lack of Enablement under 35 U.S.C. §112, first paragraph

Claims 8, 10, 11, 13, 16, 18-21, 23-25, 27, 32 and 36 are rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the enablement requirement. The Advisory Action opines that, since the claims are allegedly not supported by either a specific and substantial asserted utility or a well-established utility, one of skill in the art would not know how to make and use what is claimed.

Appellant submits that the above arguments addressing the utility requirement establish that the specification at the time of filing clearly taught how to make and use the claimed isolated nucleic acids in assays where the encoded hOCTN1 protein is expressed and screened for its ability to transport a variety of organic cations, including carcinostatic compounds. The transport activity is demonstrated in several working examples (Examples 6-8, for instance), and also is consistent with the structural information provided in the specification (e.g., in Example 2 at pages 22-23). The disclosures in the specification also enable one of ordinary skill in the art to use the claimed nucleic acids, vectors, and/or cells to make probes and/or antibodies that can be used to determine whether a patient's cancer cells express hOCTN1, and so could be treated with a carcinostatic agent that is transported by hOCTN1. Appellant therefore requests reversal of the rejection for lack of enablement.

III. Rejection for Inadequate Written Description under 35 U.S.C. §112, first paragraph

The Advisory Action maintains the rejection of claims 10, 18, 23 and 32 under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement. The Advisory Action asserts that the specification does not describe which of the one to thirty (claim 10) or one to ten (claim 32) amino acids of hOCTN1 can vary and still retain organic cation transporter function, and that "there is no description of the conserved regions that are critical to the structure and function of the genus claimed."

Claim 10 and dependent claims 18 and 23 encompass nucleic acids that encode a polypeptide having between one and thirty conservative amino acid substitutions in the hOCTN1 sequence. In the 551 amino acid sequence, this represents variants whose sequences are from 94.6% to 99.8% identical to the hOCTN1 SEQ ID NO:1 sequence, with all substitutions being

conservative ones. Claim 32 is directed to nucleic acids that encode proteins containing even fewer substitutions (*i.e.*, one to ten conservative substitutions, corresponding to a sequence identity, compared to SEQ ID NO:1, of 98.1-99.8%).

The Examiner noted during the telephonic interview of January 10, 2008, that, if the sequence and function of a protein are described in the specification (which is certainly the case for hOCTN1), variants of the protein described as being 95% or more identical to the protein and retaining the activity of the protein are generally accepted as meeting the written description requirement. This principle was affirmed in the revised Written Description Training Materials, published by the Office on March 25, 2008. As Example 10 of the Training Materials notes, when the specification describes the sequence of a protein, those of skill in the art could recognize amino acid sequences that are least 95% identical to that of the protein. This is true regardless of whether the amino acid differences are conservative or random.

Claims 10, 18 and 23 encompass nucleic acids encoding proteins that are between 94.6% (30 substitutions) to 99.8% (one substitution) identical to the sequence of the hOCTN1 protein, and all substitutions must be conservative ones. Thus, nearly all of the variants encompassed by these claims are well within the 95% variance blessed by Example 10 of the revised Written Description Training Materials. The fact that all of the substitutions must be conservative ones further limits the genus and so strengthens Appellant's position with respect to these three claims even more. Claim 32 narrows the genus down to no more than 10 conservative substitutions (i.e., at least 98.1% identity to SEQ ID NO:1), so is well under the 95% limit blessed by the revised Written Description Training Materials. Appellant therefore submits that claims 10, 18, 23 and 32 easily satisfy the written description requirement.

The rejected claims also specify that the protein variants encoded by the nucleic acids are transporters of organic cations. The Examiner alleges that there is "no description" of the sites at which variability may be tolerated and there is "no information" regarding the correlation of structure and function. Appellant respectfully disagrees. The specification describes the characterization of several significant structural features/domains of the hOCTN1 protein. For example, Fig. 1 shows a hydrophobicity plot predicting the locations of several transmembrane domains; this is discussed further at page 22, lines 11-16. A transporter consensus sequence within SEQ ID NO:2 is described at page 6, lines 6-23, as well as at page 22, lines 16-26. A

second consensus sequence is described at page 23, lines 1-14; this one is a putative ATP/GTP binding site, said to be typical for the so-called "ATP Binding Cassette type transporter" protein. The specification at page 22, lines 26-31, discloses that the sequence of hOCTN1 has four putative N-linked glycosylation sites and five putative protein kinase C phosphorylation sites, and says exactly where these sites are located in the sequence. Furthermore, the specification discloses the sequence of both the human and mouse OCTN1 proteins, enabling one of ordinary skill to align the two sequences and readily see which positions vary between the two homologs. Those non-conserved positions are presumptively able to tolerate change from the human OCTN1 sequence, particularly where the changes are all conservative substitutions. Given all of this disclosure, Appellant does not understand why the Advisory Action asserts, "**There is no description of the conserved regions that are critical to the structure and function of the genus claimed. There is no description of the sites at which variability may be tolerated and there is no information regarding the relation of structure to function.**" The Examiner plainly has not taken into account the substantial amount of such description in the specification. Given the modest scope of claim 10, and the even narrower scope of claim 32, one of ordinary skill in the art would readily recognize that Appellant was in possession of the entire genus of nucleic acids encompassed by each of these claims and their dependents.

Reversal of the rejection of claims 10, 18, 23 and 32 for lack of written description is therefore solicited.

CONCLUSION

For the reasons set forth above, Appellant respectfully requests that all of the rejections of claims 8, 10, 11, 13, 16, 18-21, 23-25, 27, 32 and 36 be reversed.

An attached Claims Appendix (viii) contains a copy of the claims under appeal.

An Evidence Appendix (ix) refers to attached Exhibits A-D.

A Related Proceedings Appendix (x) is attached as required, but contains no subject matter.

The fees in the amount of $540.00 are being paid concurrently herewith on the Electronic Filing System (EFS) by way of Deposit Account authorization. Apply any other necessary charges, or any credits, to Deposit Account No. 06-1050, referencing Attorney Docket No. 14875-0057002.

Respectfully submitted,

Date: October 27, 2008

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(viii) Appendix of Claims

8. An isolated nucleic acid encoding a polypeptide comprising the sequence of SEQ ID NO:1.

10. An isolated nucleic acid encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:1, with one to 30 conservative amino acid substitutions, wherein the polypeptide is a transporter of an organic cation.

11. An isolated nucleic acid that hybridizes under stringent conditions to a probe, wherein:

the sequence of the probe consists of the complement of SEQ ID NO:2;

the stringent conditions comprise hybridization at 68 °C followed by washing in 2 X SSC/0.1% SDS for 20 minutes at room temperature and twice in 0.1 X SSC/0.1% SDS for 20 minutes at 50 °C; and

the isolated nucleic acid encodes a polypeptide that is a transporter of an organic cation.

13. The nucleic acid of claim 11, wherein the amino acid sequence of the polypeptide comprises SEQ ID NO:1.

16. A vector comprising the nucleic acid of claim 8.

18. A vector comprising the nucleic acid of claim 10.

19. A vector comprising the nucleic acid of claim 11.

20. A vector comprising the nucleic acid of claim 13.

21. A cultured host cell comprising the nucleic acid of claim 8.

23. A cultured host cell comprising the nucleic acid of claim 10.

24. A cultured host cell comprising the nucleic acid of claim 11.

25. A cultured host cell comprising the nucleic acid of claim 13.

27. A method of producing a polypeptide, the method comprising isolating the polypeptide from the cultured host cell of claim 21.

32. The nucleic acid of claim 10, wherein the sequence of the encoded polypeptide comprises the amino acid sequence of SEQ ID NO:1, with up to 10 conservative amino acid substitutions.

36. An isolated nucleic acid encoding a polypeptide consisting of the sequence of SEQ ID NO:1.

(ix) Evidence Appendix

Exhibit A	Maiden *et al.*, *Nature*, 325:641-643 (1987)
Exhibit B	Meijer *et al.*, *J. Pharmacokin. Biopharm.*, 18:35-70 (1990)
Exhibit C	Ullrich *et al.*, *Clin. Investig.* 71:843-848 (1993)
Exhibit D	Tsuji *et al.*, *Pharm. Res.* 13(7):963-1132 (1996)

(x) Related Proceedings Appendix

There are no related proceedings.

Mammalian and bacterial sugar transport proteins are homologous

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The uptake of a sugar across the boundary membrane is a primary event in the nutrition of most cells, but the hydrophobic nature of the transport proteins involved makes them difficult to characterize. Their amino-acid sequences can, however, be determined by cloning and sequencing the corresponding gene (or complementary DNA). We have determined the sequences of the arabinose-H^+ and xylose-H^+ membrane transport proteins of *Escherichia coli*. They are homologous with each other and, unexpectedly, with the glucose transporters of human hepatoma[1] and rat brain[2] cells. All four proteins share similarities with the *E. coli* citrate transporter[18]. Comparisons of their sequences and hydropathic profiles yield insights into their structure, functionally important residues and possible evolutionary relationships. There is little apparent homology with the lactose-H^+ (LacY)[3] or melibiose-Na$^+$ (MelB)[4] transport proteins of *E. coli*.

In the bacterium *Escherichia coli* there is one H^+-linked transport system for arabinose encoded by the gene *araE*[5], and another for xylose encoded by *xylE*[6] (with gene products AraE and XylE). The linkage of sugar and H^+ translocation enables energization of nutrient uptake by the transmembrane electrochemical proton gradient[7,8].

We have isolated and sequenced the *araE* and *xylE* genes, and deduced the amino-acid sequence of each protein (Fig. 1). AraE contains 472 amino acids (relative molecular mass (M_r)51,683), and XylE contains 491 amino acids (M_r 53,607). Both are typical membrane proteins: they are very hydrophobic (about 67% nonpolar residues); their hydrophobic and hydrophilic regions are arranged alternately (Fig. 1; see also ref. 9); and their calculated M_r values are substantially higher than the values of ~36,000 for AraE[8] and 39,000 for XylE[8] measured by SDS-polyacrylamide gel electrophoresis.

The amino-acid sequence of the glucose transport protein, which does not translocate a cation, from a human hepatoma cell line (HepG2) was recently determined[1]. The reading frame

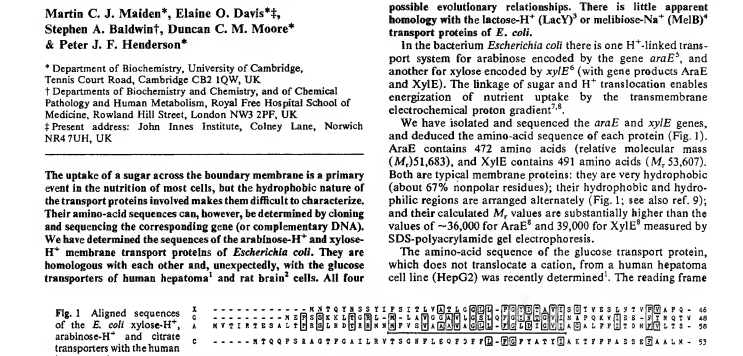

Fig. 1 Aligned sequences of the *E. coli* xylose-H^+, arabinose-H^+ and citrate transporters with the human hepatoma glucose transporter. The sequences of the glucose and citrate transport proteins were taken from refs 1 and 18, respectively. Residues are boxed in the XylE, glucose and AraE transporters if they occur more than once at an aligned position in the three sugar transporter sequences. They are only boxed in the citrate transporter if they also occur in the aligned positions of two or three of the sugar transporters. The predicted membrane-spanning regions of the glucose transporter[1] are underlined. X, XylE; A, AraE; C, citrate transporter; G, glucose transporter.

Methods. The *araE* gene is located at 61 min on the linkage map close to the *lysA* gene[5]. A restriction map of a *λplac* Mu phage inserted into the *araE* gene established its precise position by comparison with an overlapping restriction map of the *lysA* region[56]. This enabled a 2.8-kilobase (kb) DNA fragment containing the intact *araE* gene and promoter region to be cloned from a specialized *λ* transducing phage[57] into the multicopy vector pBR322. The resultant plasmid, pMM25, conferred arabinose-H^+ transport activity on appropriate recipient *E. coli* strains negative for *araE*. The *xylE* gene is located at 91.4 min on the linkage map between the *pgi* and *malB* genes[6]. A restriction map of a *λplac* Mu phage inserted into the *xylE* gene established its precise position by comparison with an overlapping restriction map of the *malB* region[58]. This enabled a 2.8-kb DNA fragment containing the intact *xylE* gene to be cloned from a λ (*xylE*) Φ (*malK'-'lacZ*) phage into the multicopy vector pBR328. The resultant plasmid, pEJ3, conferred xylose-H^+ transport activity on appropriate recipient *E. coli* strains deleted for *xylE*. The complete DNA sequence of each 2.8-kb fragment and criteria for identifying the reading frame will be described elsewhere.

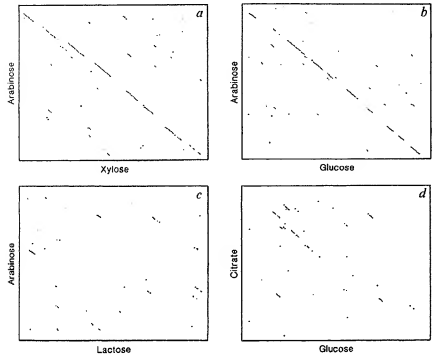

Fig. 2 Comparisons of the sequences of transport proteins using Diagon plots[10]. *a*, The arabinose-H^+ and xylose-H^+ transport proteins of *E. coli*; *b*, the arabinose-H^+ transport protein of *E. coli* and glucose transport protein of human hepatoma cells; *c*, the arabinose-H^+ and lactose-H^+ transport proteins of *E. coli*; *d*, the citrate transport protein of *E. coli* and the glucose transport protein of human hepatoma cells. The comparisons used a 21-residue segment and generated a dot if the score exceeded 240 (ref. 10).

was confirmed by the identity of parts of its sequence with peptides derived from the erythrocyte glucose transporter[1]. Its amino-acid composition was similar to that of the AraE and XylE proteins with 492 amino acids (M_r 54,117)[1]. The sequence of the glucose transporter from rat brain cells, identified and cloned using similar techniques, was virtually identical, having 492 amino acids (M_r 56,133)[2].

The sugar-transporter sequences were compared using the Diagon algorithm of Staden[10]. Several homologous regions were revealed (Fig. 2*a*, *b*) in both hydrophobic and hydrophilic regions (Fig. 1). Furthermore, their hydropathic profiles[9,11] were similar (Fig. 3); there was an hydrophilic segment, 50–65 residues long, located approximately at the mid-points of each of the sequences (Fig. 3), with some similar hydrophilic segments either side marked X in Fig. 3, and a similar number (12) of hydrophobic segments (Fig. 3).

Diagon plots drawn at the same and less stringent levels of discrimination did not reveal such homologies between XylE, AraE or the glucose transporter and the lactose[3] or melibiose[4] transporters of *E. coli* (one example is shown in Fig. 2*c*). It should be noted that the scoring matrix used by the Diagon algorithm was not devised for membrane proteins.

The sequence of the passive glucose transporter[1] is aligned with the sequences of the active arabinose and xylose transport proteins in Fig. 1. Seventy-seven residues are conserved in all three proteins (the glucose transporter has 131 identities with AraE and 142 identities with XylE; AraE and XylE have 141 residues in common). There are additional conservative substitutions[12] throughout the sequences. Consequently nearly 40% of residues can be regarded as homologous, a level sufficient to expect similar secondary and tertiary structures for all three proteins. Like many integral membrane proteins[13] these transporters lack the N-terminal signal sequences typically required for insertion of proteins through membranes[14]. The attachment site for the carbohydrate chain, Asn 45 of the hepatoma cell glucose transporter[1,15], is not conserved in the XylE or AraE proteins (Fig. 1).

The citrate-H^+ transporter[16] of *E. coli* has 431 amino acids (M_r 46,979)[17,18] and an apparent M_r of 35,000[19] (determined by SDS-polyacrylamide gel electrophoresis. Diagon comparisons revealed little homology with AraE or XylE, but some homology with the glucose transporter (Fig. 2*d*). However, all four proteins had similar hydropathic profiles (Fig. 3) with 12 membrane-spanning regions predicted by the Eisenberg algorithm[11] (Fig. 1, Fig. 3). This enabled us to align the patterns of conserved amino acids in all four transporters, particularly in the regions 30–49, 60–101, 121–149, 257–302 (Fig. 1). These patterns may be coincidental[20], but, more probably, they indicate that those residues boxed in the citrate transporter (Fig. 1) are critical for the common transport function of all the proteins.

It is interesting to locate certain conserved amino acids, in view of previous suggestions as to their roles in transport. For example, a glutamate (or aspartate) residue is responsible for H^+-translocation by subunit *c* in the F_0 moiety of H^+-ATPase[21], and Glu 325 is implicated in H^+-translocation by LacY[22], so the conservation of such residues at positions corresponding to 153, 337, 397 and 472 of XylE (Fig. 1) identifies putative H^+-translocating residues in these proteins. The mammalian transporters are not thought to translocate cations, however[23].

There is no conservation of histidines and cysteines (Fig. 1), residues implicated in the transport function of LacY[24–27]. Hence they are unlikely to have a common role in these proteins. In all three sugar transporters substrate(s) protect a thiol group against reaction with *N*-ethylmaleimide[6,28,29], a phenomenon well established in LacY and MelB (Fig. 3). It is predicted by the Robson algorithm[31] to form a β-bend in six of its eight locations. This may link two helices in a defined topology stabilized by charge-charge interactions with head groups of the lipids. Its sequence also resembles peptides recognized by mammalian cyclic AMP protein kinase[32,33].

The central hydrophilic regions of all four proteins are predicted by the Robson algorithm[31] to be α-helices, which correlates with the observation that 20% of the homologous erythrocyte glucose transporter forms non-membrane α-helix[34]. There are

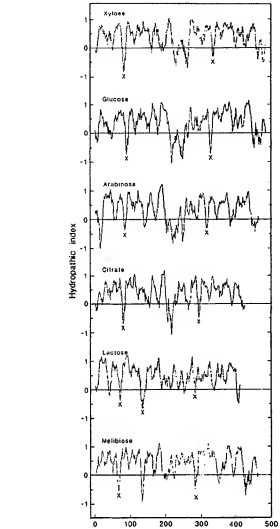

Fig. 3 Hydropathic profiles of transport proteins. Hydropathy values[9] for a window of nine amino-acid residues were averaged and plotted according to the position of the middle residue along the length of each sequence. The sequences of the indicated proteins were taken from Fig. 1 and refs 1, 18, 3 and 4. X indicates the positions of the –RXGRR– motif in each protein.

indications, such as the duplicated RXGRR motif, that the N-terminal and C-terminal halves are arranged symmetrically about this region, with six corresponding membrane α-helices on each side. An internal gene duplication event[20] is thus implied in an ancestral transporter, which then evolved to the present proteins.

The similarity between the sugar transporters from such divergent organisms as bacteria and mammals is probably too great to have arisen by convergent evolution. Gene transfer from eukaryotes to their prokaryotic symbionts remains an explanation: an example of this process has recently been described[35]. But we think it more likely that the homologies reflect functionally important parts of an ancient sugar transporter present in organisms before their divergence into prokaryotes and eukaryotes. If this is the case, homologous sugar transporters may also be found in plants, unicellular eukaryotes and other organisms. Whatever its origin, the similarity of these transport proteins suggests that a combination of biochemical, immunological and genetic techniques can now be used to exploit the advantages of working with prokaryotes to illuminate transport processes in higher organisms.

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Molecular dynamics studied by analysis of the X-ray diffuse scattering from lysozyme crystals

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It is now well established that the biological activity of proteins is related not only to their mean molecular structure, but also to their intramolecular mobility[1–4]. Nearly all techniques sensitive to dynamics have given evidence for intramolecular mobility in proteins: NMR[5,6], ESR[7], Raman spectroscopy[8,9], fluorescence quenching[10], Mössbauer spectroscopy[11], neutron scattering[12], measurements of elastic constants[13] and hydrogen–deuterium exchange[14]. The dynamics of proteins has also been approached by theoretical calculations[15,16]. We report here investigations of the atomic and molecular displacements in hen egg-white lysozyme crystals using a new technique. This technique, based on the X-ray diffuse scattering analysis (scattering out of the Bragg reflections),

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Carrier-Mediated Transport in the Hepatic Distribution and Elimination of Drugs, with Special Reference to the Category of Organic Cations

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Carrier-mediated transport of drugs occurs in various tissues in the body and may largely affect the rate of distribution and elimination. Saturable translocation mechanisms allowing competitive interactions have been identified in the kidneys (tubular secretion), mucosal cells in the gut (intestinal absorption and secretion), choroid plexus (removal of drug from the cerebrospinal fluid), and liver (hepatobiliary excretion). Drugs with quaternary and tertiary amine groups represent the large category of organic cations that can be transported via such mechanisms. The hepatic and to a lesser extent the intestinal cation carrier systems preferentially recognize relatively large molecular weight amphipathic compounds. In the case of multivalent cationic drugs, efficient transport only occurs if large hydrophobic ring structures provide a sufficient lipophilicity–hydrophilicity balance within the drug molecule. At least two separate carrier systems for hepatic uptake of organic cations have been identified through kinetic and photoaffinity labeling studies. In addition absorptive endocytosis may play a role that along with proton-antiport systems and membrane potential driven transport may lead to intracellular sequestration in lysosomes and mitochondria. Concentration gradients of inorganic ions may represent the driving forces for hepatic uptake and biliary excretion of drugs. Recent studies that aim to the identification of potential membrane carrier proteins indicate multiple carriers for organic anions, cations, and uncharged compounds with molecular weights around 50,000 Da. They may represent a family of closely related proteins exhibiting overlapping substrate specificity or, alternatively, an aspecific transport system that mediates translocation of various forms of drugs coupled with inorganic ions. Consequently, extensive pharmacokinetic interactions can be anticipated at the level of uptake and secretion of drugs regardless of their charge.

KEY WORDS: carrier-mediated transport; organic cations; cationic drugs; hepatobiliary elimination; hepatic distribution; drug interactions; structure-pharmacokinetic relationship; multiplicity in carrier proteins.

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INTRODUCTION

The liver performs a variety of functions to maintain whole body homeostasis, such as uptake and processing of nutrients absorbed from the intestine, cholesterol metabolism and bile formation, metabolic conversion of exogenous and endogenous substances by the phase I cytochrome P450 system and by the phase II synthetic conjugation reactions, and many others. Hepatic function is essential for disposition of many drugs and toxic agents (see Fig. 1 for the liver structure). Compounds can be taken up from the bloodstream into the liver and subsequently metabolized, excreted in bile, or transported back to the blood. Relatively lipophilic drugs enter the hepatic parenchymal cells by passive diffusion in their undissociated form (1,2). For more hydrophilic compounds, for instance, glycosides and agents with a negative or positive charge at physiological *p*H, specialized uptake processes such as carrier-mediated transport are generally necessary for penetration into the cells.

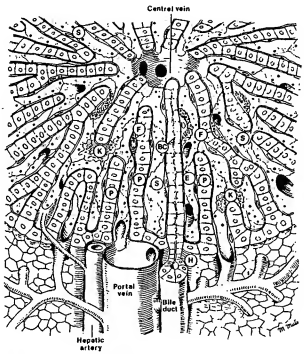


Fig. 1. Schematic figure of the three dimensional structure of hepatic tissue. BC, bile canaliculus; H: Hering canal; E: endothelial cell; F: fat-storing cell; P: parenchymal cell; K: Kupffer cell; S: fenestrated sinusoid. [Adapted from M. Muto (178).]

A large category of drugs consists of compounds with one or more quaternary or tertiary amine functions. Quaternary ammonium groups with a nitrogen linked to four carbon atoms are permanently positively charged (Fig. 2). The tertiary amines potentially are also cations since, at a pK_a of the basic group of ≥ 7.5, at physiological pH the major part of the molecules is protonated. Based on their charge the organic cations are generally divided into monovalent and bivalent cations. Among the monoquaternary drugs are anticholinergic, antineoplastic, and antihelmintic agents. The bisquaternary compounds represent predominantly neuromuscular blocking agents. The tertiary amines include a wide variety of drugs, including local anaesthetics, psychotropic amines, sympathomimetic and sympatholytic agents as well as antihistaminics.

Cationic drugs can pass membranes by carrier-mediated transport and such processes have been characterized in the kidney tubular cells (3-5) explaining secretion of basic drugs from blood into the urine, in intestine as related to secretion of organic cations from blood into the intestinal lumen (6), in the choroid plexus as a mechanism to remove cationic compounds from the liquor to blood (5) as well as in the liver mediating hepatobiliary excretion of cationic drugs (1,5,7,8).

Structure-kinetic relationship has been studied with series of monovalent (9-12) and bivalent organic cations (8,13,14). In all of the excretory organs a two-step carrier-mediated transport seems to be involved: uptake into the cells and excretion from the cells. At both levels saturation

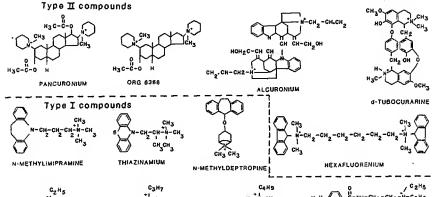


Fig. 2. Chemical structures of organic cationic model compounds. Ethyl-procainamide and tributylmethylammonium represent Type I compounds of relatively low molecular weight. d-Tubocurarine and pancuronium represent Type II compounds being relatively large molecular weight compounds with the cationic centers enclosed in or close to bulky ring structures.

and competitive phenomena have been described (15–22). The hepatic transport of monovalent organic cations, often with a spatial separation of the cationic group from the aromatic ring structure (Type I compounds, see Fig. 2), has traditionally been characterized using the model compound procainamideethobromide, PAEB) or its ^4N-acetylated derivative (5,15–21) but also, more recently, with some aliphatic methyl-ammonium compounds (9,10,28). Endogeneous substrates for this transport system may include choline and thiamine (23,24) as well as nicotinamide riboside (25). Transport of organic cations was shown to be sensitive to temperature changes, anoxia, and metabolic inhibitors (16,22,26,27). Multivalent organic cations only seem to undergo significant hepatobiliary transport if the presence of the cationic groups is masked by bulky ring structures (1,8,14) and uptake occurs via a process that differs from that of the Type I compounds (17). They represent the Type II class of organic cations (Fig. 2). At the canalicular pole of the cell the bulky organic cations and the PAEB-like agents may share one carrier-mediated secretion process as evidenced by mutual interactions at that level (17,28). In some cases net canalicular transport can be promoted by bile salts through choleretic effects, ion pair formation or binding to biliary micelles (1,29) but this is certainly not a general rule, especially if uptake into the cells is rate limiting in the hepatobiliary transport (30).

STRUCTURE-PHARMACOKINETIC RELATIONSHIP OF MONOVALENT ORGANIC CATIONS

Several reviews have been published on the hepatic (8,27), renal (4,31), and intestinal (6,32) transport of organic cations. In general, quantitatively important hepatobiliary elimination is restricted to drugs with a relatively large molecular weight (33). A so-called "molecular weight threshold" was proposed above which the biliary excretion of drugs becomes appreciable ($>10\%$ of the dose). For monovalent organic cations the molecular weight threshold was suggested to be 200 ± 50 Da in the rat (12). However, molecular weight indirectly reflects other physicochemical characteristics, such as lipophilicity. In this context Schanker (5) noted that the chemical structures of organic cations that are secreted into bile have the following characteristics in common: a positively charged quaternary ammonium group at one end of the molecule and one or more nonpolar ring structures at the opposite end. Several later studies (10,11,34) indicated that the lipophilicity of the organic cations appears to be a better parameter for the prediction of hepatobiliary transport. However it should be noted that the above-mentioned data are related to transport from blood to bile, not allowing a discrimination between the hepatic uptake and the biliary

excretion processes. Data from Neef *et al.* (9) showed that even hydrophilic, low molecular weight cations (< 200 Da) are substantially accumulated in the liver within 10 min; no clear relation with lipophilicity was observed with regard to hepatic uptake. In contrast, the biliary excretion of the monovalent cations increased with increasing lipophilicity (9,10), thus suggesting that lipophilicity is predominantly a prerequisite for efficient transport from the hepatocyte into the bile. Yet the extent of lipophilicity alone cannot fully explain hepatobiliary transport profile of organic cations, as was shown in a study with thiazinamium and its very polar sulfoxide metabolite, which are equally well excreted in bile (35). This suggests that not the lipophilicity *per se* but rather the balance between hydrophilic and hydrophobic properties plays a crucial role. Even the tricyclic ring structure including the polar sulfoxide group may provide sufficient opportunity to interact with the supposed carrier sites. With the presence of the positively charged group, the presence of a planar ring structure some distance from the cationic moiety may represent a minimal condition for drug-carrier interaction. In addition, the differences in the hepatobiliary transport of two stereoisomers of oxyphenonium indicate that the spatial structure may also influence transport (36).

A study with 14 organic cations (Fig. 3) showed that while biliary excretion becomes more important with increasing lipophilicity, no evident relation with lipophilicity exists for renal clearance (10). These observations are in line with the idea of Rennick (31) that only the positive charge of the molecule is important for tubular secretion. Quaternary amines are transported more efficiently than the tertiary ones which in turn are transported more effectively than the secondary ones (4). Yet recent data indicate that for organic cations and anions several subsystems with overlapping substrate specificity exist in the kidney (3). This implies that more specific molecular features may play a role in renal excretion. This is also indicated by the differences in the tubular secretion of several stereoisomers of organic cations (3) and by the different transport of MPP^+ and MPTP in renal brush border membrane vesicles (37).

With respect to the secretory function of the intestine, studies from the group of Lauterbach (38) demonstrated that the permeation of quaternary ammonium compounds across jejunal mucosa proceeds much faster from the blood side into the luminal compartment than in the absorptive direction, indicating intestinal secretion (38,39). The secretion of organic cations occurs by a saturable mechanism and can be inhibited by structurally related compounds (32). The luminal system, secreting the organic cations against the membrane potential, was inhibited by anaerobiosis, whereas the uptake at the basolateral membrane was not affected (38). Similar to hepatic elimination, intestinal secretion of organic cations increases with increasing

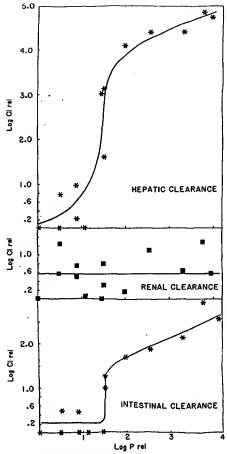


Fig. 3. Relation between lipophilicity (measured by partition between octanol-Krebs buffer and expressed relative to the value of tetraethylammonium) and carrier-mediated clearance of 14 monovalent organic cations (drugs with a quaternary ammonium group) via biliary, intestinal, and renal routes in the rat. The clearance values depicted are corrected for passive fluxes (glomerular filtration etc.), blood flow limitation, and protein binding and indicate carrier-mediated transport in the organs. Between log $P = 1$ and log $P = 2$, biliary clearance increases about 1000 and intestinal clearance about 100 times in contrast to renal clearance that shows no such correlative pattern.

lipophilicity of the compound (10,38). In accordance with the phylogenetic origin of the liver from a gut diverticulum, the secretory system in the intestine may be more closely related to that in the liver than to that in the kidney (6).

STRUCTURE–PHARMACOKINETIC RELATIONSHIP OF BIVALENT ORGANIC CATIONS

In general, nondepolarizing neuromuscular blocking agents are characterized by two cationic centers in the molecule. The elucidation of their kinetics is of major importance to achieve muscle relaxation of predictable intensity and duration of action (40–42). Among the major determinants in the pharmacokinetics, and thus the course of the muscle-relaxing effect, are the distribution and the elimination of the muscle relaxant. Due to their high hydrophilicity, the distribution volume of the muscle relaxants is approximately limited to that of the extracellular fluids (43). Yet some association with specific tissue components occurs. Muscle relaxants have a great affinity for the acid mucopolysaccharides including chondroitin sulfuric acids of the connective tissues, where high tissue concentrations for various muscle relaxants have been found (44–48).

Biotransformation, renal and hepatic excretion are the main processes contributing to the elimination of muscle relaxants from the body. In man, biotransformation of nondepolarizing muscle relaxants, in general, exerts little influence on the elimination (43). Many relaxants are excreted unchanged and if metabolities are formed, they account for only small proportions of the administered dose (49–51). An exception in this respect is atracurium, which is rapidly degraded to inactive breakdown products partly by Hoffman elimination and by direct reactions with nucleophiles (glutathione, cysteine) in plasma (52,53). These nonbiological methods of degradation occur at physiological temperature and *p*H (52,53). In addition, enzymatic ester hydrolysis contributes to the degradation of atracurium (53).

In general, urinary excretion forms the major elimination pathway for these muscle relaxants. This holds especially for gallamine (54), alloferine (55), metocurine (56), pancuronium (57), pipecurium (58,59), and chandonium (60) and is generally accompanied with a relatively long duration of action. Hepatic elimination presents an alternative route of elimination for many relaxants. If significant biliary excretion occurs for a given agent, often distribution to the liver is rapid and this process of rapid removal from the circulation alone may terminate the action of the compound (61). Vecuronium is predominantly excreted in bile (61) and its efficient hepatic uptake from the circulation is a major determinant of its short duration of

action (61,62). (Fig. 4). Hexafluronium is excreted mainly in bile in rat and in man (63), while the biliary route is also an important way of elimination for d-tubocurarine (28,56). Rapid uptake of stercuronium in the liver is the major factor determining the short duration of action in the rat (44). In the cat the liver was shown to accumulate substantial amounts of pancuronium and its congeners dacuronium and Org 6368 (49). It is obvious that impaired liver or kidney function, often, more or less, affects the elimination rate of the relaxants. The clinical implications of this impaired organ function with respect to the elimination of curare-like agents have recently been reviewed by Booij (64).

With respect to the molecular features determining the route of elimination, a molecular weight threshold of 500–600 Da has been proposed for hepatobiliary excretion of bivalent organic cations (14). Similar to the monovalent cations, lipophilicity seems to be an important factor in determining the elimination pathway of bivalent organic cations (13,65,66). The importance of the lipophilicity is clearly reflected in the different hepatic disposition of d-tubocurarine and its less lipid soluble trimethyl derivative metocurine (66) as well as in the very dissimilar kinetics of vecuronium and pancuronium (67,68), which differ in only one methyl group at one of

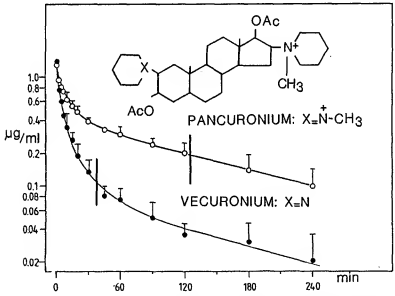
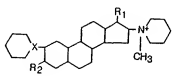


Fig. 4. Plasma concentrations of vecuronium and pancuronium after 0.1 mg/kg intravenously, in man. Vertical bars bisecting the plasma curves indicate plasma concentrations at the time point of 50% recovery of muscle contraction. Modified from Sohn *et al.* (51).

the amine centers (see Fig. 4). The higher molecular weight threshold for bivalent cations in hepatobiliary transport, as compared to the monovalent cations, might reflect the amount of apolar elements in the molecule that is in general required to balance the bivalent ionic character. This may hold especially for the canalicular excretion, which occurs against the inside negative membrane potential. Thus the lipophilicity-hydrophilicity balance of the type II organic cations appears to be an important factor determining hepatic transport both at the uptake and secretion levels. However, some remarkable exceptions exist. For instance, stercuronium is rapidly taken up by the liver in spite of a small octanol/Krebs partition coefficient (44,69). In addition, the low molecular weight hydrophilic bivalent cations decamethonium and hexamethonium appear to accumulate in the liver to a substantial degree (70), whereas biliary output of these compounds is minimal (14,70). It is possible that for such compounds an endocytotic uptake mechanism is an alternative route. Such a process was recently described for the diquaternary fluorescent dye lucigenin that by virtue of its concentrated positive charges may induce absorptive endocytosis. This process leads to an endosomal/lysosomal sequestration without appreciable biliary output (71).

The hepatobiliary transport of several structurally related but physicochemically distinct steroidal muscle relaxants (Fig. 5) was recently studied in isolated perfused rat liver (72). Marked differences were observed in the overall hepatobiliary transport of the four muscle relaxants under study. The most hydrophilic compound, pancuronium, showed the lowest net transport from perfusate to bile. Conversely, the most lipophilic relaxant, vecuronium (the monoquaternary analog of pancuronium) showed the most efficient hepatobiliary transport. Vecuronium was very effectively taken up in the liver and within 2 hrs more than 60% of the dose was excreted in



	X	R_1	R_2	MW_{cation}	Oct./Krebs part. coeff.
Vecuronium	N	$OCOCH_3$	$OCOCH_3$	557	2.56
3-Hydroxyvecuronium	N	$OCOCH_3$	OH	515	0.032
Org 6368	N^+-CH_3	H	$OCOCH_3$	514	0.0145
Pancuronium	N^+-CH_3	$OCOCH_3$	$OCOCH_3$	572	0.0033

Fig. 5. Structural formulas and physicochemical data of some steroidal organic cations (peripheral muscle relaxants).

bile. The bisquaternary relaxants Org 6368 and 3-hydroxyvecuronium showed an intermediate behavior, with effective uptake in the liver but only modest excretion in bile. Similar to pancuronium, the amount in the liver versus time curves indicated that hepatic storage in a deep compartment might be involved in the hepatic disposition of steroidal muscle relaxants.

Cardiac glycosides are potent inhibitors of the hepatobiliary transport of bivalent organic cations (8,17,24,73). Hepatic uptake of the three relatively hydrophilic muscle relaxants was also strongly reduced by K-strophanthoside, whereas the uptake of the more lipophilic vecuronium was only partly affected at the dose ratio used. Similar to vecuronium, a modest effect of K-strophanthoside was also observed in studies with the rather lipophilic bivalent cation hexafluronium (73). This may imply that passive diffusion is partly involved in the hepatic uptake of these lipophilic muscle relaxants. Examples of other extremely lipophilic cations are methyldeptropine (8,76) and rhodamine B (74). The latter compound has been shown to enter the liver parenchymal cells by passive noncarrier-mediated diffusion in spite of the presence of a cationic center (75). Alternatively, the affinity of such relatively lipophilic cations for the supposed common carrier site may be so high that the cardiac glycoside fails to compete successfully during hepatic uptake.

PHARMACOKINETIC MODELING AND ANALYSIS OF HEPATIC ORGANIC CATION TRANSPORT

The plasma disappearance and biliary excretion rate versus time curves obtained after a single injection of 1 mg of the steroidal muscle relaxants in the perfusion medium of isolated perfused rat livers were fitted with the computer program DIFFIT. This program enables simultaneous fitting of plasma disappearance and biliary excretion rate curves, yielding the best model to explain these independently measured profiles. In contrast with the traditional nonlinear curve-fitting methods, this method is based on defining input and output of the drug, and subsequent simulation of disappearance and appearance patterns by a numerical approach. Iterative changing of the parameters, such as rate constants and volumes of distribution, is followed by comparison of the experimental data with the calculated curves, using least squares regression analysis. The combination of rate constants and distribution volume in the various differential equations were adapted until a minimal sum of squares was attained, using the Simplex procedure for an optimal simultaneous change of parameters. The program offers the possibility to test several multicompartment models without the necessity of deriving the complex equations by integration of sets of differential equations, as performed in the traditional compartment analysis.

Apparent volumes of distribution V_2 and V_3 were estimated from the amounts in the various compartments under simulated steady state conditons. After the fitting procedure the optimal model is discriminated by application of the F-ratio test. In the fitting procedure corrections were applied for loss of drug and circulating volume by sampling of perfusate. Fitting of experimental versus simulated curves was assumed to be optimal when the change in sum of squares was less than 0.01%. The experimental data were weighted according to the y^{-2}-method. The program was developed in cooperation with Zernicke Science Park, University of Groningen, as part of the software package "MW PHARM".

A kinetic analysis revealed that the hepatobiliary transport of the steroidal muscle relaxants was best described by a three-compartment model with elimination from the peripheral compartment V_2 and storage in a deep compartment V_3, connected to V_2 (see Fig. 6). Rather than molecular weight (12), the lipophilicity of the muscle relaxants influenced the kinetic parameters of the hepatobiliary transport. The biliary clearance (Cl_{20}) and the initial hepatic uptake (Cl_{12}) showed a positive relationship with the lipopholicity, confirming that hepatobiliary transport of these organic cations is highly dependent on the hydrophobic character of the compounds (8,10,11). In addition, net hepatic uptake of the muscle relaxants (rate of distribution to the liver) is markedly influenced by the liver to plasma transport (hepatic efflux). The tendency for hepatic efflux, expressed as the k_{21} value, increases with decreasing lipophilicity. This phenomenon can be envisoned if the dissociation of the relatively lipophilic compounds from

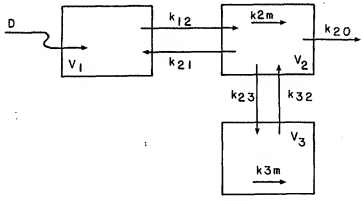


Fig. 6. Compartmental model for pharmacokinetic analysis of the hepatic uptake, storage, and biliary excretion of organic cationic drugs. Rate constants for transport between the compartments are indicated (fraction per unit of time), k_{2m} and k_{3m} represent rate constants for metabolism (de-acetylation) in compartment 2 and 3, respectively.

the carrier sites at the outer side of the membrane were the rate-limiting step in the hepatobiliary efflux process.

In analogy with the classical muscle relaxant d-tubocurarine (66,77–79) the kinetic analysis revealed an intracellular deep compartment. The affinity of the muscle relaxants for this intracellular distribution compartment, expressed as the k_{23}/k_{32} ratio, is inversely related with lipophilicity. However, it should be realized that the absolute amount of drug that is accumulating in the deep compartment is also determined by the fraction of the dose that can penetrate into the hepatocyte (80,81). The size of this fraction is determined by the k_{12}/k_{21} ratio, a factor that increases with increasing lipophilicity. Although the combined data indicate that lipophilicity has a major influence on several stages of the hepatobiliary transport of these organic cations, it is important to note that the net uptake of the muscle relaxants (indicated by the k_{12}/k_{21} ratio) is of special importance, inasmuch as the rate of the various subsequent transport steps is dependent on the concentration of the drug in the hepatocyte cytoplasm as the driving force for these processes.

HEPATIC TRANSPORT MECHANISMS FOR MONOVALENT ORGANIC CATIONS

Two compounds have been used most frequently as model compounds in the study of hepatobiliary transport of organic cations: procainamide ethobromide (PAEB) and its N^4-acetyl derivative (APAEB). Experiments with these compounds have been performed using several techniques such as rats *in vivo* (9,16–18,21,23,24,82), isolated perfused livers (17,30), rat liver slices (16,20,22), isolated hepatocytes (15,20,83), and liver membrane vesicles (84). An important contribution to a better understanding of hepatic transport mechanisms for organic actions was made by Schanker and co-workers. They showed that after intravenous injection of PAEB into the rat, the drug reaches concentrations in the bile far exceeding the concomitant plasma values (26). The biliary excretion was saturable and could be suppressed by structurally related compounds (21). Uptake into liver slices was blocked by anoxia and a number of metabolic inhibitors (22). These combined data indicated that carrier-mediated mechanisms are involved in hepatobiliary transport of organic cations, as was later on supported by other studies (16,19). Liver fractionation studies (23,24,30) pointed to two concentrative steps in the transport from blood to bile (8), indicating that energy-requiring transport systems are involved. Other mechanisms than passive distribution should be involved since concentration ratios of free PAEB between cytosol and plasma and between bile and cytosol considerably exceed the equilibrium value that is anticipated if distribution had

taken place according to the transmembrane potential (23,24). For the hepatic uptake step this was corroborated in a study with freshly isolated rat hepatocytes, which indicated that PAEB is taken up by a primary or secondary active carrier-mediated mechanism (15).

A new approach to carrier identification was introduced by the application of the technique of photoaffinity labeling in the field of hepatic transport. The concept of photoaffinity labeling (Fig. 7) is based on ligands that have an inherent affinity for a binding site but also contain a photosensitive functional group that, when photoactivated, is capable of forming a covalent bond at or near the binding site (85-91). Subsequent identification of the binding polypeptide is possible by concomitant introduction of a radioactive marker in the photolabile substrate. Unlike electrophilic affinity labels, their association with the recognition site will ordinarily be reversible until photolysis is initiated. The recent introduction of high-energy flash photolysis thus might enable investigations of time-dependent transport processes (86).

In order to identify potential transport polypeptides for these low molecular weight cations, a photolabile derivative of the classical model compound PAEB was synthesized: azidoprocainamide methoiodide (APM). Determination of the octanol/Krebs partition coefficient and binding to albumin of APM revealed that the introduction of the photolabile azido group did not markedly change the physicochemical parameters as compared to its parent compound PAEB. Experiments in isolated perfused rat liver demonstrated that APM is efficiently taken up in the liver and excreted into bile, partly in the form of metabolites (87).

The inhibitory effect of other cations such as tributylmethylammonium on the hepatobiliary transport of APM suggests that carrier-mediated

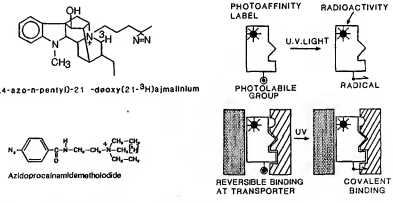


Fig. 7. Principle of photoaffinity labeling and photoaffinity probes of organic cations to label potential carrier proteins in hepatocyte plasma membranes.

mechanisms are involved, as was demonstrated for PAEB (15-17,20,22). Kinetics of the uptake of APM in isolated rat hepatocytes revealed that both saturable and nonsaturable processes are involved. The saturable part of the uptake was best described by two uptake systems (V_{max1} = 80 pmol/min · 10^6 cells, $K_{m1} = 3\ \mu M$ and $V_{max2} = 130$ pmol/min · 10^6 cells, $K_{m2} = 100\ \mu M$). APM uptake was Na^+-independent, but replacement of bicarbonate in the incubation medium by chloride decreased the hepatic uptake. The mechanism of this bicarbonate effect, that occurs at an unchanged extracellular *p*H, remains to be clarified. An effect on secondary mechanisms involved in the driving of the uptake may underly the observed phenomenon.

In order to investigate the specificity of the hepatic uptake systems for APM, the influence of several classes of hepatic model compounds on the uptake of APM was studied. The results show that all the mono- and bivalent organic cations studied substantially decreased the uptake of APM into the liver cell. In presence of anionic and uncharged compounds the uptake of APM was not inhibited. These data indicate that APM is taken up by transport systems differing from the systems for anions and uncharged compounds. The complete lack of effect of high concentration of APM on the uptake of bulky organic cations indicates that APM is taken up by a system distinct from the uptake mechanisms for such agents, but that on the other hand the high molecular weight (Type II) compounds exhibit considerably affinity for the supposed uptake system for low molecular weight organic cations. Probably only carrier occupation without net transport occurs.

HEPATIC TRANSPORT MECHANISMS FOR BIVALENT ORGANIC CATIONS

Hepatobiliary transport mechanisms for bivalent cations have predominantly been studied using the classic muscle relaxant d-tubocurarine as the model compound. Besides the chemical difference in the number of cationic centers in the molecule, bivalent cations also show a marked difference in hepatobiliary transport, as compared with the monovalent cations. In the case of the bivalent cations, the hepatic uptake can be strongly inhibited with relatively low concentrations (1 μM) of cardiac glycosides (8,17,73). The biliary excretion step however seems not to be affected by these agents (73). In contrast, uptake of monovalent cations is not influenced by cardiac glycosides (15,17). The effect of the cardiac glycosides does not appear to be linked directly to their inhibitory effect on the Na^+K^+-ATPase, since the concentrations in which they inhibit the uptake of bivalent cations are too low to affect the Na^+K^+-ATPase of rat liver to a significant degree

(19,93). In addition K-strophanthoside and digitoxin are much more potent inhibitors of bivalent cation uptake than ouabain, while their potency to inhibit this ATPase is quite similar (94). The mechanism of uptake of steroidal cations has been investigated in isolated rat hepatocytes (80), using vecuronium as a model compound (81). Determination of initial uptake velocity at different vecuronium concentrations demonstrated that vecuronium uptake into the hepatocyte occurs by both a saturable (K_m = 15 μM, V_{max} = 181 pmol/min · 10^6 cells and a nonsaturable (k = 1.10 pmol/min · 10^6 cells · μM) process. This implies that in the *in vitro* situation the saturable component is mainly responsible for the hepatic uptake, since plasma concentrations in the rat usually do not exceed 5 μM.

The uptake of vecuronium satisfies the other criteria for carrier-mediated transport. The uptake is inhibited by structurally related compounds, is temperature-dependent, and decreased by various metabolic inhibitors. The inhibitory effect of SH-reagents indicate that sulfhydryl groups may be located at the active site of the transport system. Uptake into the liver of d-tubocurarine and metocurine is probably the rate-limiting step in the overall hepatobiliary elimination process as indicated by the low intracellular (cytoplasmic) concentration. However, this is not a general rule for the bivalent cations.

Determination of the unbound fractions of some cationic steroidal muscle relaxants in Krebs-albumin solution, in the cytosolic fraction of liver homogenate, and in bile, enabled a rough estimation of the concentration gradients across the sinusoidal and the canalicular membrane. The data indicated that major differences exist in the net sinusoidal uptake of pancuronium, Org 6368, and vecuronium which increased in the order pancuronium < Org 6368 < vecuronium. The calculated chemical gradients for vecuronium and Org 6368 were 16 and 1.6, respectively, and that for pancuronium less than 1 (see Fig. 8). Yet the cytosol–plasma concentration ratios of these agents did not exceed the values that would be attained by passive equilibration according to the membrane potential. At the canalicular site "uphill" transport of the muscle relaxants into bile against an electrochemical gradient occurs. Thus some kind of active transport is inferred, which appears to be more efficient for Org 6368 and vecuronium than for pancuronium. The nearly equal bile–cytosol concentration ratios for Org 6368 and vecuronium suggest that the differences in biliary excretion rate between these two substances are not caused by differences in membrane transport at the canalicular level, but rather by an unequal extent of binding within the liver cells. Since vecuronium and Org 6368 strongly differ in lipophilicity, it is evident that even a modest hydrophobicity is sufficient for effective canalicular transport of organic cations, which is in line with data of Neef *et al.* (35). The limited hepatobiliary transport of pancuronium

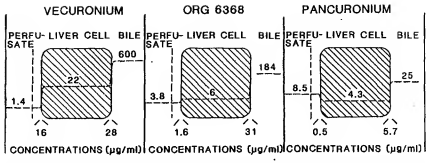


Fig. 8. Hepatobiliary concentration gradients of the Type II organic cations in rat liver. The unbound concentrations in perfusate, hepatic cytosol, and bile are indicated together with the calculated concentration gradients across the sinusoidal and canalicular membranes (81).

however appears to be due to inefficient net transport, both at the sinusoidal and at the canalicular membrane and apparently its hydrophobicity is below a critical level for proper carrier-mediated transport at these levels.

SUBCELLULAR DISTRIBUTION OF ORGANIC CATIONS IN RELATION TO HEPATIC STORAGE AND EXCRETION RATE

Accumulation in the liver may, apart from efficient membrane transport, also be explained by extensive intracellular binding (8,23,24,71). Quaternary agents are appreciably bound to the particulate fraction after liver homogenization (23,24,66). Liver subfractionation studies (10) as well as electron microscopy of d-tubocurarine molybdate precipitates in liver sections strongly suggest association with lysosomes in hepatocytes (66,78,79) (Fig. 9). The accumulation in these organelles can be partially inhibited by chloroquine (79). Association of organic cations with lysosomes presents a likely explanation for the persistent hepatic storage of these compounds in other studies (28,44,77,95). The mechanism of the lysosomal accumulation of this type of cation remains to be elucidated. Proton-driven antiport of organic cations from the cytosol into the lysosome, followed by intralysosomal trapping caused by the protonation of the tertiary amine group of the drug in this acidic compartment, might play a role (96). Triggering by organic cations of aspecific fluid phase endocytosis and subsequent vesicular transport to the lysosomes might form an alternative explanation (71). This mechanism has been proposed for the renal accumulation of the strongly basic aminoglycosides (97,98). Receptor-mediated co-endocytosis by non-covalent binding of d-tubocurarine to α_1-acid glycoprotein was recently excluded as a possible mechanism for lysosomal accumulation (99).

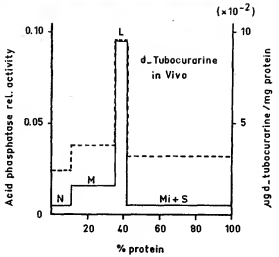


Fig. 9. Lower part: Subcellular distribution of d-tubocurarine and acid phosphatase in the liver 15 min after iv injection of 2 mg/kg of ^{3}H-d-TC into rats (*in vivo*) and after the addition of ^{3}H-d-TC to cold liver homogenates (*in vitro*). Specific activities are indicated ($n = 3$). The broken line indicates distribution of d-TC and the solid line that of acid phosphatase. Fractions are represented by their protein content cumulatively from left to right in the order of their isolation. N, M, L, and S indicate the nuclear, mitochondrial, lysosomal, and supernatant fraction, respectively. Upper part: Electronmicroscopy of liver sections of d-tubocurarine-treated rats. After fixation the liver tissue blocks were treated with ammonium molybdate, a substance that gives an electron-dense precipitate with curare-like drugs. Small electron-dense particles are found in and around lysosome-like bodies in the hepatic parenchymal cells. The dense deposits are not found in livers from untreated control animals.

Pancuronium, Org 6368, and vecuronium liver homogenate subfractionation (100) indicated that the steroidal muscle relaxants were predominantly accumulated in the mitochondrial fraction (95). Accumulation in mitochondria has been earlier demonstrated for various other mono- and bivalent cations (47,101–103) and might be explained by passive equilibration according to the mitochondrial membrane potential (47). Alternatively, active carrier-mediated uptake of organic cations in mitochondria might be involved (103). In this context a potential interaction with the endogenous substrate NAD^+ has been inferred (101–103). In comparison with the classical muscle relaxants d-tubocurarine and metocurarine (66,78,79) accumulation in the lysosomal fraction seemed to be of less importance in the case of the steroidal muscle relaxants and decreased relative to the association with the mitochondrial fraction if the liver load was increased. The particle-cytosol concentration ratio of Org 6368 was significantly higher than the values for vecuronium and pancuronium and might explain the effective intracellular storage of Org 6368, as observed in the isolated perfused rat liver experiments (69) as well as in cat *in vivo* (49).

MULTIPLICITY IN HEPATIC UPTAKE MECHANISMS FOR DRUGS

The classical view on hepatic uptake mechanisms states that there are three separate transport pathways, depending on the charge of the substrate (anions, cations, and uncharged compounds) (5,7,104). For several reasons this traditional scheme may require revision. As discussed above, the effect of cardiac glycosides on the uptake of cations discloses a marked difference in uptake between bivalent and monovalent cations. Similarly, for organic anions at least two uptake systems are inferred (105–107). With respect to organic anions one roughly differentiates between sodium-independent (bilirubin, BSP) and sodium-dependent (bile acids, fatty acids) uptake systems. Yet also for bile salts and for fatty acids, which are both taken up by sodium-dependent mechanisms, separate systems are demonstrated (108), indicating that more than two carrier-mediated systems are involved in the uptake of organic anions (92). It is conceivable that the diversity in organic anion uptake systems is related to the presence of various naturally occurring anionic substrates such as bile salts, bilirubin, and fatty acids. An analogous situation appears to hold for the uncharged compounds, which include many naturally occurring substrates such as steroid hormones. Data from uptake studies with various uncharged compounds (cardiac glycosides, steroid hormones) suggest the existence of more than one uptake system for uncharged compounds (109,110). These combined data indicate

that the liver is equipped with multiple uptake systems with limited substrate specificity.

On the other hand the concept of separate uptake pathways for organic anions, cations, and uncharged compounds is challenged by the occurrence of major interactions between representatives of these classes of compounds. Traditionally, many studies on hepatic transport dealt with bile salts. Kinetic data show that bile salts can not only inhibit the hepatic uptake of other organic anions (107,111–114) but also of the uncharged cardiac glycosides (110,115,116), cyclopeptides (112,117,118), and bivalent cations (29,69). High bile salt plasma concentrations have been shown to prolong the effect of muscle relaxants, both in animal and man (66,119), most probably due

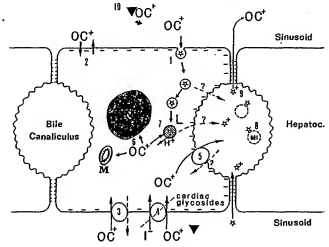


Fig. 10. Mechanisms for uptake of organic cations into hepatocytes. Adsorptive endocytosis (1) leads to accumulation of the cation in endosomes, lysosomes and other vacuoles. Lipophilic cations can also enter the cell by passive diffusion (2), others undergo carrier-mediated uptake. At least two carrier systems are involved: one for small cations (3) and one for larger cationic compounds (4). At least one carrier system mediates biliary excretion of organic cations (5). Direct uptake from the cytoplasm takes place into nuclei (6), into mitochondria and into lysosomes (7). Accumulation in lysosomes may, apart from aspecific fluid-phase endocytosis at the plasma membrane, occur from the cytoplasm via antiport with protons within the lysosomes. Direct transport of drug from lysosomes to bile could occur extremely slowly by exocytosis. Biliary excretion involves carrier-mediated transport, possibly by antiport with inorganic ions. Binding to mixed biliary micelles may facilitate net transport into the bile canaliculus (8). Small organic cations can probably directly pass the tight junctional channels that have a negative charge (9). Intrahepatic dissociation from α_1-acid glycoprotein (triangle) occurs in the sinusoids (10).

to uptake inhibition of the muscle relaxant by the bile salts (69). Also the reverse inhibition has been reported: The uptake of bile salts in the hepatocyte can be decreased in presence of steroid hormones (113,120), cardiac glycosides (105,121), cyclopeptides (112,122), cholecystographic agents (112), and various other compounds (123–125). Furthermore, mutual uptake inhibition between representatives of these different groups of substrates has been reported (15,78,115,116,122,125–127,131). Cardiac glycosides inhibit the hepatic uptake of bivalent cations (17,73) and bivalent cations have been shown to decrease the uptake of ouabain into isolated hepatocytes (115). The occurrence of these mutual interactions might be explained by the existence of uptake systems with broad substrate specificity (88,115,125,128). A general transport system for bulky organic compounds with multiple ring structures, irrespective of charge, presents an attractive explanation for the observed interactions (1,2,88,129). The detoxification function of the liver may benefit from such a system with broad substrate specificity.

In conclusion, based on the results of the aforementioned studies, the hepatic disposition of organic cations can be schematically pictured as depicted in Fig. 10.

IDENTIFICATION OF POTENTIAL CARRIER PROTEINS

In addition to kinetic studies, attempts were made to identify potential transport polypeptides for several categories of drugs (Fig. 11). Table I summarizes studies on identification of potential carrier proteins in rat liver, using various affinity labeling techniques. From this table it is evident that the majority of the identified binding polypeptides for the different model compounds have apparent molecular weights of about 48,000 and/or 55,000, irrespective of the technique used. In combination with kinetic data the results of these studies have been interpreted as supporting the hypothesis of uptake systems with broad substrate specificity (91,129). With respect to the identification of carrier polypeptide(s) for uncharged compounds such as cardiac glycosides and organic cations, only limited information is available. A M_r 50,000 polypeptide was labeled with a photolabile ouabain derivative (90). Since ouabain is taken up in the liver cell by a Na^+-independent carrier-mediated mechanism (115,130,131), the labeled polypeptide would be anticipated to represent this Na^+-independent system. Whether or not the M_r 50,000 binding polypeptide described by Petzinger *et al.* (90) is the same as the M_r 48,000 or 54,000 protein that is labeled with bile salt derivatives, remains to be established. It is tempting to relate the two major labeled proteins to the Na^+-dependent and Na^+-independent transport systems for organic anions. Bilirubin and BSP, that are taken up

POTENTIAL CARRIER-PROTEINS FOR HEPATIC TRANSPORT OF DRUGS

Mol Weight	48 kDa	48–49 kDa	50 kDa	54–55 kDa (OABP)	54–55 kDa	55 kDa	100 kDa	110 kD (BTL, $\alpha_1\beta$)
Protein-isolation	—	+	–	+	–	+	+	+
Photo-aff. label	+	+	+	+	+	–	+	–
Antibody	–	+	–	+	–	+	+	+
Reconstitution	–	–	–	–	–	–	+	+
Co-factors	H^+	Na^+	HCO_3^-	CL^-	OH^-	–	HCO_3^-	H^+ ?
Sinusoidal/Canalicular	S	S	S	S	S	S	C	S
References	1,87,88, 89,146	89,90,91, 129,133, 135,136, 141,168, 173,174	1,88, 89,146	139,172	88,90, 91,117, 147,162, 173,174, 175	92,138, 170,171	143,144, 163	137,140, 169
Substrates	Organic Cations (Type I)	Bile Acids Phalloidin	Organic Cations (Type II)	BSP (GSH) Bilirubin	BSP Bile Acids Steroids	BSP (GSH) Bilirubin ICG	Bile Acids	BSP Bilirubin ICG Rifamycin Nicotinic Acid

Fig. 11. Experimental work reported on potential carrier proteins responsible for hepatic and biliary excretion of organic anions and other drugs. Estimated molecular weight, use of photoaffinity techniques, preparation of antibody with measurement of its effect on uptake, reconstitution experiments performed, possible cofactors or driving forces established, and binding or transport of substrates are indicated. The scheme suggests separate carrier processes; however, some may turn out to be identical (for instance, 1st, 2nd, 3rd and 4th, 5th and 6th from left to right).

by a Na^+-independent mechanism, appear to label predominantly a M, 55,000 polypeptide, whereas bile acid derivatives label both the 48,000 and 55,000 species. Since mutual competitive inhibition has been demonstrated for BSP and Na^+-independent bile acid uptake (107,111), the M, 55,000 polypeptide might be a likely candidate for the Na^+-independent BSP transporter. In this concept the polypeptide with apparent molecular weight of 48,000 might be related to the Na^+-dependent bile acid uptake system.

Photoaffinity labeling with the Type I organic cation azidoprocainamide methoiodide (APM) was performed on isolated hepatocytes and sinusoidal membrane fractions. The experiments revealed two membrane polypeptides with apparent molecular weight of 48,000 and 72,000 that are involved in binding with APM. Differential photoaffinity labeling in presence of substrates that inhibit the uptake of APM suggested that these polypeptides might be involved in the hepatic uptake of APM (87).

Photoaffinity labeling with a photolabile derivative of N-propyldeoxyajmalinium (NPDA), a bulky monovalent quaternary amine that was used as a Type 2 model compound was recently performed (146). Physicochemical parameters, such as albumin binding and lipophilicity (expressed as octanol/Krebs partition coefficient) of NPDA appeared to be highly comparable with the steroidal muscle relaxant vecuronium. Studies in isolated perfused rat liver demonstrated mutual uptake inhibition for both

Table I. Binding Polypeptides in Hepatocyte Plasma Membranes as Identified by Different Affinity Labeling techniques

Parent compound	App. MW ($\times 10^{-3}$)	Technique[a]	Preparations used: Intact cells	Preparations used: Plasma membranes	Authors (Reference no.)
Procaine	21.4	PAL	—	V	Levy *et al.*, 1977 (132)
BSP	60	AC	—	V	Reichen and Berk, 1979 (170)
	55	PAL	—	V	Wolkoff and Chung, 1980 (172)
	37/35		—	V	Lunazzi *et al.*, 1982 (169)
	56	AC	—	V	Stremmel *et al.*, 1983 (171)
Bilirubin	60	AC	—	V	Reichen and Berk, 1979 (170)
	56	AC	—	V	Stremmel *et al.*, 1983 (171)
	54/48	PAL	V	—	Buscher *et al.*, 1986 (88)
NAP-taurine	54/43	PAL	V	V	Cheng and Levy, 1980 (168)
	54	PAL	V	—	Von Dippe and Levy, 1983 (133)
Taurocholate	52/48	PAL	—	V	Kramer *et al.*, 1982 (147)
	54	PAL	V	V	Von Dippe and Levy, 1983 (133)
	54/48	PAL	V	V	Wieland *et al.*, 1984 (91)
Cholate	54/50	AL	—	V	Ziegler *et al.*, 1984 (173)
Taurodehydrocholate	54/50	AL	V	V	Ziegler *et al.*, 1984 (174)
DIDS	54/50	AL	V	V	Ziegler *et al.*, 1984 (174)
Phalloidin	54/48	PAL	V	V	Wieland *et al.*, 1984 (91)
Antamidine	54/48	PAL	V	V	Wieland *et al.*, 1984 (91)
Oleate	40	AC	—	V	Stremmel *et al.*, 1985 (108)
Ouabain	50	PAL	—	V	Petzinger *et al.*, 1986 (90)
α-Amanitin	54/48	PAL	V	V	Krönckе *et al.*, 1986 (117)
Cyclosporine	54/50	PAL	V	V	Ziegler and Frimmer, 1986 (175)
Ajmalinium	48	PAL	V	—	Buscher *et al.*, 1986 (88)

[a] PAL: photoaffinity labeling; AL: affinity labeling; AC: affinity chromatography.

compounds. Furthermore the hepatic uptake of the monovalent cation NPDA was inhibited by taurocholate and by K-strophanthoside, thus showing a striking parallel with vecuronium-like compounds (147).

It is crucial to prove that the identified polypeptide not only binds the drug but that it is also able to translocate the substance across the membrane. Indirect approaches to relate photoaffinity labeling to transport function include comparison with photoaffinity labeling in cells that lack the transport function under study, e.g., AS 30 D hepatoma cells (134) or studying the rate of uptake after photoaffinity labeling (135). An alternative approach is to study the effect of antibodies, raised against the purified protein, on drug transport. Recently several groups reported inhibitory effects of antibody preparations on the uptake of organic anions (108,136–139). Stremmel and Berk (138) and Wolkoff *et al.* (139) described an antibody against a BSP/bilirubin binding protein with apparent molecular weight of 55,000. The antibody prepared by Stremmel and Berk inhibited uptake of BSP and

bilirubin but did not affect the uptake of taurocholate, cholate or oleate in isolated hepatocytes (138). Since the antibodies of both groups showed only weak cross-reactivity, further comparative studies are necessary to elucidate structural and immunological similarities. Levy *et al.* (136) reported an antibody against a M, 54,000 membrane protein, which specifically inhibited Na^+-dependent taurocholate transport in isolated hepatocytes but had no effect on BSP transport. Integration of these data suggests that at least two proteins with apparent molecular weights in the range of 55,000 are involved in organic anion transport (92).

Besides immunological studies, reconstitution of transport with the isolated proteins might provide evidence for their physiological function. Sottocasa *et al.* (140) performed reconstitution experiments with the potential carrier protein bilitranslocase. The results showed transport of BSP, which could be driven by a negative membrane potential, a process that could be imagined in relation to sinusoidal or canalicular secretion from the cell. Von Dippe *et al.* (141) reported reconstitution in liposomes with a M, 54,000 polypeptide that exhibited several characteristics of Na^+-dependent taurocholate transport. However, substrate specificity of the reconstituted transport systems was not investigated in either study.

Only limited data are available with respect to the polypeptides involved in bile-canalicular transport. By photoaffinity labeling of rat liver snips, a preparation in which functional polarity is preserved, Fricker *et al.* (142) identified a bile salt-binding polypeptide with an apparent molecular weight of 100,000 at the canalicular membrane. Inhibition of bile salt transport in canalicular plasma membrane vesicles by an antibody against this M, 100,000 polypeptide indicated that this protein is involved in canalicular excretion of bile salts (143). Transport could be reconstituted in liposomes (144) and was shown to be basically different from the sinusoidal Na^+-dependent uptake system.

With regard to potential canalicular transport polypeptides for organic cations, Kamimoto *et al.* (145) reported data on a canaliculnr membrane protein with an apparent molecular weight of 170,000, which might be the efflux pump for amphipathic cationic anticancer drugs in hepatocytes. The membrane protein has two internal ATP binding sites, and ATP-dependent transport of anticancer drugs in canalicular membrane vesicles was inhibited by verapamil and other drugs. More data are available on membrane proteins that are involved in the hepatic uptake process for amphipathic cationic drugs.

Subsequent photoaffinity labeling with the photolabile NPDA-derivative of isolated cells revealed two plasma membrane binding polypeptides with apparent M,s of 48,000 and 50,000. Differential photoaffinity labeling studies demonstrated decreased incorporation of radioactivity in the plasma membrane polypeptides in the presence of amphipathic cations

(vecuronium, pancuronium, d-tubocurarine, quinidine, and verapamil) and in the presence of uncharged compounds (K-stophanthoside, ouabain, digitoxin), compounds that all inhibit the uptake of NPDA in cells. These data suggest that the identified binding polypeptides may be involved in the hepatic uptake of the amphipathic cation (88, 146).

DRIVING FORCES IN THE HEPATIC TRANSPORT OF DRUGS

The general idea of uphill transport raises the question on the energization and driving forces for these processes. In the case of taurocholate numerous studies indicated that the carrier-mediated hepatocellular uptake is driven by an inwardly directed Na^+-gradient (105,111,124,148–150). The process is characteristic of a Na^+-coupled secondary active transport driven by the Na^+-gradient that is maintained by the activity of the basolateral Na^+K^+-ATPase. Several studies with basolateral membrane vesicles showed facilitation of taurocholate uptake by the negative intracellular potential, suggesting that cotransport occurs with more than one Na^+ ion (142,148–151). Recent data suggest that only certain conjugated bile acids are transported by this system (154) and alternative forms of uptake may be important for other types of bile acids (105,124,153). Cholate transport is dependent upon the presence of chloride, which might be selectively needed as a discharging counteranion (155). Data from a study of Blitzer *et al.* (156) suggest hydroxyl/cholate exchange related to the Na^+/H^+ exchanger that has been identified in the basolateral membrane (157). The uphill cholate transport might be explained by a "tertiary active transport" model in which the Na^+K^+-ATPase would ultimately drive the Na^+/H^+ exchange, which in turn would drive hydroxyl/cholate exchange (156). Alternatively, the results can also be explained by H^+/cholate cotransport, and may therefore in principle occur by passive noncarrier-mediated diffusion of the unchanged protonated molecule.

Hepatic uptake of anions like BSP and iodipamide occurs by Na^+-independent mechanisms. In the uptake of both compounds an electrogenic component might be involved and appears to be related to an inwardly directed chloride gradient (130,158–160). Analogous chloride dependency was demonstrated in the carrier-mediated uptake of *p*-aminohippurate in rat renal basolateral membrane vesicles (161). In a study with short-term cultured rat hepatocytes a mechanism involving Cl^-/organic anion exchange was proposed (160). Studies with vesicles of sinusoidal and canalicular membranes revealed exchange mechanisms with inorganic anions (sulfate, bicarbonate, chloride) that appear to be involved in hepatobiliary transport of xenobiotics (162,163). The opportunities would provide a flexible and dynamic combination of transporting systems for organic compounds.

Information on potential driving forces for organic cation transport is scarce. Even with respect to possible Na^+-dependency of organic cation uptake, no clear picture emerges from the available data. Studies on the uptake of morphine, nalorphine (164), and thiamine (23) suggest that this process is partly Na^+-dependent, whereas the uptake of PAEB appears to be Na^+-independent (15). The inside negative membrane potential might provide the driving force for the uptake of organic cations by facilitated diffusion, a mechanism that has also been proposed for the organic cation transport across the basolateral membrane in the kidney (3,31). The uptake of vecuronium also appears to be independent of the Na^+-concentration in the extracellular medium. On the contrary the uptake of vecuronium exhibits anion dependency. Both cotransport of the cationic substrate with inorganic anions to maintain electroneutrality (44,89,165) and carrier-mediated uptake of (electroneutral) ion pairs (1,2,86,153,166) with vecuronium, formed at the fluid/lipid interface, might explain the observed anion dependency. In sucrose medium, a medium that increases the negative transmembrane

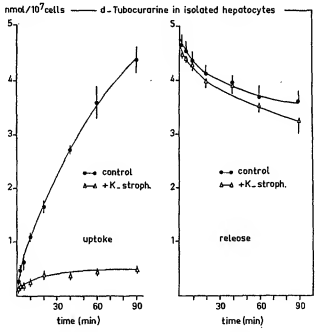


Fig. 12. Uptake of d-tubocurarine during incubation with isolated hepatocytes that can be strongly inhibited by K-strophanthoside at a concentration of 10^{-5}M. In contrast (right) the cardiacglycoside in the same concentration does not affect release from hepatocytes preloaded with d-tubocurarine.

potential, the uptake of vecuronium was decreased instead of increased, supporting the concept of electroneutral uptake of the muscle relaxant.

As discussed before, both kinetic and photoaffinity-labeling studies provided evidence that the liver is equipped with transport systems with broad overlapping substrate specificity (88,91,129). The effective inhibition of d-tubocurarine by cardiac glycosides (17,73) might therefore be related to a common uptake pathway for uncharged compounds including drugs with masked or neutralized charges (see Fig. 12).

The stimulating effect of ion pair-forming anions such as iodide on the hepatic uptake of organic cations also suggests transport as an electroneutral species (1,167). In the kidney organic cation transport across the brush border membrane occurs by a cation/proton antiport, resulting in net electroneutral uptake (31,118,166). Whether an analogous mechanism might

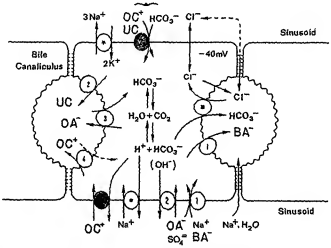


Fig. 13. Coupling between gradients of inorganic ions with carrier-mediated transport of organic anions (OA^-), bile acids (BA^-), organic cations (OC^+) and uncharged compounds (UC) in the hepatocyte. Inorganic ion-pumps include electrogenic Na^+/K^+ exchange, Na^+/H^+, $OH^-/SO_4^{=}$-antiport (sinusoidal) and Cl^-/HCO_3^- and $SO_4^{=}/HCO_3^-$ antiport (canalicular). Na^+-coupled bile acid transport (1) and OH^-/OA^--antiport (2) at the sinusoidal level and HCO_3^-/OA^--antiport at the canaliculi are carrier systems for organic anions. Organic cations of Type I are taken up by System 1 and Type II compounds may share a transport System 2, also accommodating uncharged compounds. In the latter system ion pair formation may play a role. At the canalicular level antiport of organic cations with inorganic cations is tentatively assumed. The transport processes at sinusoidal level have more overlapping substrate specificity than the projected four canalicular carrier systems. [Adapted from G. Hugentobler and P. J. Meier (162).]

operate in the hepatobiliary transport or in the lysosomal accumulation of organic cations is presently under investigation (96).

The potential coupling between inorganic ion gradients in the hepatobiliary system and drug transport is tentatively depicted in Fig. 13. Confirmation of this scheme requires further experimentation in a multi-experimental approach with techniques ranging from intact hepatocytes to plasma membranes and reconstituted transport systems.

CONCLUSIONS

The knowledge of mechanisms for carrier-mediated transport of drugs in the organism is necessary for the interpretation of pharmacokinetic patterns in the practical situation:

1. Carrier-mediated transport in the uptake into and secretion of charged drugs from cells in the eliminating organs is in principle saturable so that nonlinear kinetic patterns are to be expected.
2. Carrier-mediated transport in drug elimination and distribution potentially can be reflected in mutual interactions between drugs and also implies potential interactions with endogenous substrates such as bile acids.
3. The relative contribution of the liver, kidney, and intestines to carrier-mediated secretion of drugs in the body can be roughly predicted on the basis of the chemical structure and physicochemical characteristics.
4. Drugs can influence each others distribution and elimination patterns through interactions on the carrier level but also by changing concentration gradients of inorganic ions representing driving forces for transmembrane transport.
5. On the basis of chemical structure and charge, potential transport interactions of drugs can in principle be identified. Caution should be exercised here since drugs can be present in various forms (dissociated, undissociated, complexed with counterions etc.) and therefore can be transported by various carrier systems at the same time. As discussed, a general aspecific transport system for amphipathic drugs in the liver cannot be excluded. Such a transport system may for instance underly the multiple kinetic interactions in the distribution and elimination of cardiac glycosides such as digoxin and basic drugs such as quinidine and verapamil (176,177) that have also been reported at the hepatic level.
6. The involvement of the various carrier mechanisms in the total cellular transport of a given compound is determined by its concentration, affinity for the carrier proteins, and the presence of competing substrates.

7. Distribution to the liver through carrier-mediated uptake is often a rapid and quantitatively important process that in itself can lead to termination of the action after single doses of drugs.
8. Carrier-mediated transport is not restricted to the plasma membranes of the secretory cells: Various organelles within the cells can accumulate drugs via similar translocation mechanisms. This can lead to persistent storage of such drugs, changes in organelle function, or local toxicity.

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Renal transport mechanisms for xenobiotics: chemicals and drugs

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Summary. Using the stopped flow tubular lumen or peritubular capillary microperfusion method, the apparent K_i values of a large number of organic anions and cations against the respective transport systems were evaluated. Thereby the luminal transport system for monocarboxylates (lactate), the contraluminal and luminal transport systems for dicarboxylates (succinate), sulfate, and hydrophobic organic cations (tetraethylammonium or N^1-methyl-nicotinamide), as well as contraluminal transport system for hydrophobic organic anions (para-aminohippurate, PAH) were characterized and their specificity determined. There is a partially overlapping substrate specificity between the PAH, dicarboxylate, and sulfate transport systems but also between the PAH and organic cation transport system. Xenobiotics and their metabolites are transported mainly by the organic anion (PAH) and organic cation transport systems. To test the complicated interactions possiblc a shot injection/urinary excretion method with simultaneous measurement of the intracellular concentration was developed. With this approach it is possible to evaluate (a) whether a substrate is net secreted or net reabsorbed, (b) whether interference with other substrates occurs, (c) whether interference takes place at the luminal or contraluminal cell side, and (d) whether *cis*-inhibition or *trans*-stimulation is the predominant mode of interaction. Finally, it will be discussed which ability a substrate must have to penetrate the cell membrane via a transporter, through the lipid bilayer, or both.

Key words: Transport interaction – Organic anions – Organic cations – Sulfate – Dicarboxylates

Most xenobiotics are excreted by the kidney, either directly or after metabolic transformation [23]. The site of transport is the proximal renal tubule. During transtubular transport the substances must cross two cell membranes, the contraluminal and the luminal cell membrane. In this overview we discuss three principle questions: (a) how many transport systems are involved in the transport of xenobiotics, (b) what is their specificity, and (c) whether they interact.

Fortunately a large set of data (Table 1) allows the establishment of a quantitative relationship between chemical structure and interaction with the respective transporters. These data were obtained on the rat kidney in situ by stopped flow microperfusion of the tubular lumen [14] or peritubular capillaries [6] and by initial flux measurements. For each transport system a test substance was identified and the inhibitory potency (apparent K_i values in mmol/l) of respective xenobiotics against these test substances was evaluated. As depicted in Fig. 1, the characteristics of the luminal transport system for monocarboxylates (lactate) [14, 15], contraluminal and luminal transport systems for dicarboxylates (succinate) [12, 16], sulfate [4, 17–19], and hydrophobic organic cations [tetraethylammonium (TEA) or N^1-methyl-nicotinamide (NMeN)] [5, 25, 27], as well as the contraluminal transport system for hydrophobic organic anions (para-aminohippurate, PAH) [20–23, 26] were well defined. The luminal transport system for PAH was investigated only in kidney membrane vesicles [9, 11] and not in the tubule in situ. Since the transport systems for hydrophobic organic anions and cations are the principle mechanisms for the transport of xenobiotics, the main emphasis should be put on these systems. There exists overlapping specificity of the

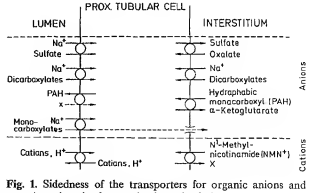

Fig. 1. Sidedness of the transporters for organic anions and organic cations in the proximal renal tubule

Table 1. Groups of chemical substances interacting with contraluminal anion and cation transporters in the proximal renal tubule

Transporter for hydrophobic organic anions (PAH) [20–24, 26, 30, 31]	Transporter for dicarboxylates [12, 16, 20, 22]	Transporter for sulfate/oxalate [17–20, 22, 24]	Transporter for organic cations [24, 25, 27, 30, 31]
Monocarboxylates		Sulfate esters	Aliphatic amines
Dicarboxylates	Dicarboxylates	Dicarboxylates	Mono-quaternary compounds
Substituted benzene analogs	Substituted benezene analogs	Substituted benzene analogs	Bis-quaternary compounds
Benzoates	Benzoates	Sulfonates	Catecholamines
Phenols	Phenols	Aminosulfonates	Piperidines
Aldehydes		Disulfonates	Piperazines
Phenoxy compounds		Salicylate analogs	Azepines
Sulfamoyl compounds		Sulfamoyl compounds	Pyridines
β-Lactam antibiotics	β-Lactam antibiotics		Quinolines
Phenolphthaleins	Phenolphthaleins	Phenolphthaleins	Imidazoles
Sulfonphtaleins	Sulfonphtaleins	Sulfonphtaleins	Thiazoles
Fluoresceines	Fluoresceins	Fluoresceins	Thiazides
			Guanidines
Dipeptides			Dipeptides
N-acetyl-*N*-benzoyl-amino acid analogs		Diphenylaminc carboxylates	Cyclophosphamides
Glutathione conjugates			
Cysteine conjugates			
Cyclic nucleotides			
Eicosanoids			
Corticosteroids			Corticosteroids
Bile acids	Bile acids	Bile acids	

PAH transport system with the transport systems for dicarboxylates, sulfate, and lactate, which has been discussed previously [7, 28]. Roughly speaking, most xenobiotics are transported by the transport system for hydrophobic anions (PAH), some by that for hydrophobic cations, and some by both. If a xenobiotic is transported by the PAH transport system, it might be also transported by that for dicarboxylates, sulfate, and/or lactate. To avoid misunderstanding it must be stated that the organic "anion" (PAH) and organic "cation" (NMeN, TEA) transporters do not see the ionized form of the substrate [29], indicating that the interaction of the substrate with the respective carriers occurs by hydrogen bonds rather than by electrostatic interaction. Furthermore, substrates that cannot be ionized at all, such as corticosteroids, interact with both transporters [31].

Transport system for hydrophobic organic anions (para-aminohippurate)

The specificity of the PAH transport system is broad and partially overlapping with those of the dicarboxylate and sulfate transporters (Table 1). The PAH transport system accepts hydrophobic molecules with a negative ionic or a partial negative charge of electron-attracting side groups. The hydrophobic domain must have a minimal length of 4 Å (Fig. 2) [7]. Thus, in the fatty acid series interaction starts with molecules longer than valerate [21]. The PAH transport system also interacts with substrates which have two ionic or partial negative charges, preferentially with a charge distance of 6–7 Å [20, 22]. The hydrophobic domain can have a size of up to 10 Å and may be located at least partially outside a line connecting the two charges [7]. The role of hydrophobicity for interaction with the transporter has been shown not only for substituted benzoates [22] but also for a large group of dipeptides [23] and imidazoles [30]. Furthermore, the strength of the ionic charge is a determinant for the interaction with the PAH transporter. Thus, the interaction of substituted benzoates and phenols with the PAH transporter rises in inverse relation to their pK_a values (Fig. 12 and 13 in [22]). As side groups with electron attracting properties serve Cl, Br, NO_2, CHO, and SO_2-NH_2. However, OH, NH-CO-CH_3, and O-C_2H_5 groups also interact with the carrier presumably by hydrogen bond formation. In the same direction points the fact that many corticosteroid hormones interact with the contraluminal PAH transporter [26], whereby the spatial orientation of OH groups is important. Thus, hydrophobicity, negative charge strength (electron-attracting power), and hydrogen bond formation in favorable spatial orientation determine interaction with the PAH transporter. Metabolic transformations of xenobiotics and drugs, such as hydroxylation, N-acetylation, N-

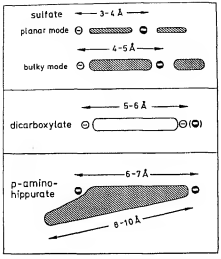

Fig. 2. Structural models of substrates for the three types of contraluminal anion-transporting systems in the rat kidney [7]. The sulfate system accepts short mono- or bivalent anions: (a) planar molecules with COO^- residues, flat hydrophobic domains, and second ionic or partial negative charge at 3–4 Å distance and (b) bulky molecules with SO_3^- residues, bulky, or flat hydrophobic domain and a charge separation of 4–5 Å. The dicarboxylate system accepts bivalent anions with a charge distance of 5–6 Å, whereby one charge might be a partial electronegative charge. The PAH system accepts monovalent anions if they bear a hydrophobic moiety of a minimal length of 4 Å and substrates that have only electron-attracting side groups. It also accepts bivalent anions with a charge distance of 6–7 Å. For some inhibitors the hydrophobic area is longer than the spacing between the two charges. ▨ Hydrophobic domain; ⊖ negative ionic charge obligatory; ⊖ negative ionic charge favorable, but electron attracting or hydrogen-bond forming groups sufficient

benzoylation, and conjugation with sulfate, glucuronate, taurine, or glutathione (with further processing), render a molecule acceptable by the PAH transporter [24]. Specific inhibitors for the contraluminal PAH transport system are benzoylbutyrate, apalcillin, and 2-nitro-4-azido-phenylalanine, while probenecid is only a relatively specific inhibitor since it interacts also – albeit weakly – with the contraluminal sulfate/oxalate transporter [28].

Transport systems for organic cations

The contraluminal transport system for organic cations accepts positively charged molecules according to their hydrophobicity. Thus, primary, secondary, and tertiary aliphatic and aromatic amines as well as quaternary N-compounds interact the stronger the more hydrophobic they are [3, 25]. This rule, however, does not hold for anilines and analogs [27]. With local anesthetics [3] and with heterocyclic compounds and their analogs [27] an inverse relationship between pK_a and apparent K_i has been demonstrated. Dependence on pK_a and hydrophobicity of the contraluminal transport system for organic cations resembles closely the PAH transporter. In addition, steroid hormones interact with the contraluminal transport system for organic cations, whereby the pattern of OH groups favorable for interaction is different to that for the PAH transporter [31]. Thus, similarly to the contraluminal PAH transporter, interaction with the contraluminal organic cation transporter depends on hydrophobicity, charge strength (electron-donating power), and hydrogen bond formation [30].

The luminal transport system for organic cations is an exchange system which can also be driven by H^+ ions. Its specificity is different from that of the contraluminal transport system for organic cations; some substrates interact luminally much less than contraluminally, and others interact luminally with greater potency [5]. Whether the different affinities play a role for the direction of net transport of organic cations is to be established.

Bisubstrates

A considerable number of xenobiotics fulfill the specificity requirements for the organic anion (PAH) transporter and for the organic cation transporter [30, 31]. As expected, they interact with both transporters and, as shown for amiloride and cimetidine, are also transported by both transporters. They extend from zwitterions, for example, dipeptides, to steroid hormones [31]. Interestingly, also the thiazide diuretics and furosemide analogs belong to this category of substrates [32]. Again, the close similarity between organic anion and organic cation transporters becomes evident. This may give the impression that the common denominator for both transport systems is hydrophobic interaction with the substrate and hydrogen bond formation in a spatial arrangement. Hydrogen-bond strength may be high enough to stabilize the complex of substrates with the transporters but low enough to allow rapid substrate dissociation from the transporter. Even sites with ionic charge may be involved in hydrogen bond formation, a hypothesis which is supported by the fact that neither carrier senses whether its substrates are ionized in the bulk phase or not [29]. The fact that many xenobiotics interact with the transporter for organic anions and organic cations augments the possibility of interaction considerably. Thus a method is urgently needed to test the quality, quantity and site of interaction in an "untouched" kidney preparation.

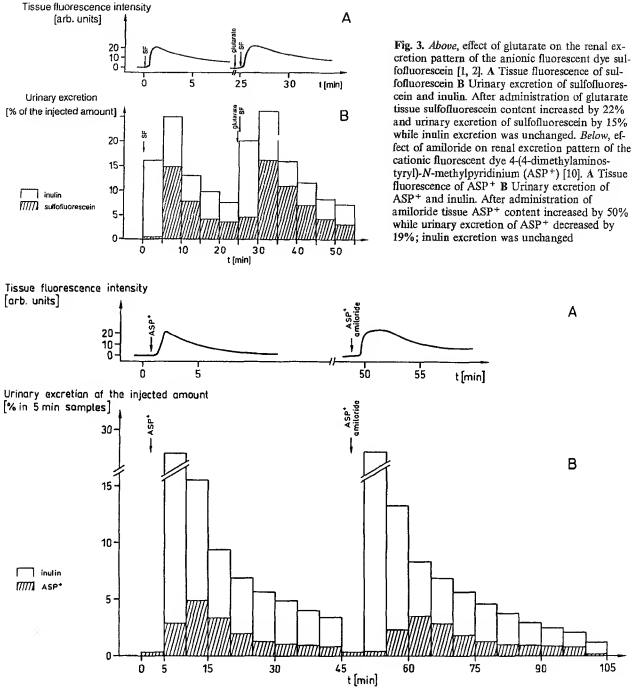

Fig. 3. *Above*, effect of glutarate on the renal excretion pattern of the anionic fluorescent dye sulfofluorescein [1, 2]. **A** Tissue fluorescence of sulfofluorescein **B** Urinary excretion of sulfofluorescein and inulin. After administration of glutarate tissue sulfofluorescein content increased by 22% and urinary excretion of sulfofluorescein by 15% while inulin excretion was unchanged. *Below*, effect of amiloride on renal excretion pattern of the cationic fluorescent dye 4-(4-dimethylaminostyryl)-*N*-methylpyridinium (ASP^+) [10]. **A** Tissue fluorescence of ASP^+ **B** Urinary excretion of ASP^+ and inulin. After administration of amiloride tissue ASP^+ content increased by 50% while urinary excretion of ASP^+ decreased by 19%; inulin excretion was unchanged

Interactions as revealed in the "untouched" kidney

The most informative interactions of some substances have been found in the rat kidney in situ during osmotic diuresis and bolus injection of test and interfering substances into the jugular vein [1, 10]. Thereby urinary excretion of a fluorescent or F-containing test substance relative to inulin was determined in the absence or presence of an interfering substance. This approach allows evaluation of whether a test substance is only filtered or, in addition, secreted or reabsorbed, and of the way in which secretion or reabsorption of the test substances is influenced by interfering substances. By simultaneous measurement of surface fluorescence or ^{19}F nuclear magnetic resonance signal on the exposed kidney in situ the cellular content of the test substance can be monitored continuously. By

combining changes in secretion or reabsorption with simultaneous increase or decrease of tissue content it can be determined whether an interference takes place luminally or contraluminally, and whether *cis*-inhibition or *trans*-stimulation is involved.

Figure 3 presents two examples [2, 10]. In the upper part, urinary excretion of the anionic dye sulfofluorescein (the "visible PAH") relative to inulin is shown in the absence and presence of glutarate. Correcting for protein binding, sulfofluorescein is filtered and secreted. After glutarate administration the urinary excretion of sulfofluorescein increases. Simultaneously the tissue content of sulfofluorescein also increases. The two changes together indicate that glutarate stimulates the contraluminal uptake of sulfofluorescein by *trans*-stimulation [13]. The lower part of Fig. 3 depicts excretion of the cationic dye 4-(4-dimethylaminostyryl)-*N*-methylpyridinium [10]. As regards protein binding the dye is filtered and reabsorbed. After simultaneous administration of amiloride the urinary excretion of the dye decreases, but the tissue content increases. These changes together indicate that reabsorption across the luminal membrane increases by *trans*-stimulation through amiloride. It is evident that this method may reveal many unexpected interactions in the transport of various xenobiotics.

When does transport occur over the carrier and when through the lipid bilayer?

The question of the relationship between K_i values of a substrate against PAH and NMeN transport and its own transport parameters (K_m, J_{max}) can be approached by the stopped flow peritubular capillary perfusion method only if the substrate is available in radiolabeled form of high specific activity. Large initial flux rates, no indication of saturability, and lack of effects of inhibitors (probenecid, TEA) indicate that the substance penetrates the cell membrane by diffusion through the lipid bilayer. Usually this coincides with high log-octanol distribution values [30], but it is also seen with zwitterions, where the effect of charge is apparently neutralized [31]. If substrates, traversing the cell membrane predominantly by diffusion, show definitive K_i values against PAH and/or NMeN transport, penetration is likely to occur, in addition, through the respective transporters. One might suppose that in such a case transport through the carriers is irrelevant. This, however, need not be the case, as exemplified by the handling of drugs by tumor cells. Here inward diffusion through the cell membrane lipid bilayer and outward transport through a carrier determines the intracellular concentration of antitumor drugs [8]. Increasing outward transport at unchanged diffusion lowers the intracellular drug concentration, an event responsible for multidrug resistance.

From the results of many transport studies which we have performed in recent years we suggest that the K_i values which we have obtained are based on a competitive type of inhibition. Indeed K_i and K_m have coincided whenever we have tested it. With decreasing K_i (K_m) values the initial transport velocity (at low substrate concentration) increases, reaches a maximum, and decreases again even with lower K_i. In the latter case the substrate acts as an inhibitor and is hardly or not at all transported. We have obtained definitive transport parameters of all radiolabeled substrates available to us except glycocholic acid and furosemide, which exhibit high unspecific binding to the cell membrane preventing proper transport measurements.

The data presented in this overview indicate that it is possible to predict the transporters with which the various xenobiotics interact. By the shot injection/urinary excretion tissue concentration method it is also possible to reveal whether transport interaction in the "untouched" kidney takes place. In this context changes in the cellular concentration of xenobiotics may reveal insights into their potential toxic effects. Noninvasive methods may also be feasible for clinical application.

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Review

Carrier-Mediated Intestinal Transport of Drugs

Akira Tsuji[1,2] and Ikumi Tamai[1]

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Recent advances in the field of carrier-mediated intestinal absorption of of amino acids, oligopeptides, monosaccharides, monocarboxylic acids, phosphate, bile acids and several water-soluble vitamins across brush-border and basolateral membranes are summarized. An understanding of the molecular and functional characteristics of the intestinal membrane transporters will be helpful in the utilization of these transporters for the enhanced oral delivery of poorly absorbed drugs. Some successful examples of the synthesis of prodrugs recognized by the targeted transporters are described. Functional expression of the multidrug resistance gene product, P-glycoprotein, as a primary active transporter in the intestinal brush-border membrane leads to net secretion of some drugs such as anticancer agents in the blood-to-luminal direction, serving as a secretory detoxifying mechanism and as a part of the absorption barrier in the intestine.

KEY WORDS: carrier-mediated transport; transporter; intestinal absorption; amino acid, oligopeptide, glucose; hexose; monocarboxylic acid; lactic acid; short-chain fatty acid; phosphate; bile acid; vitamin; intestinal secretion; active efflux pump; p-glycoprotein; multidrug resistance.

INTRODUCTION

It has long been believed that synthetic drugs are absorbed through the epithelium from the gastrointestinal tract by a simple diffusion mechanism, which would favor lipophilic and unionized drugs. However, there are direct and indirect evidences for participation of carrier-mediated membrane transport mechanisms, where several hydrophilic compounds seem to be absorbed efficiently via such specialized transporters. Therefore, utilization of the intestinal epithelial transporters to facilitate the absorption of appropriately modified drugs seems to be an attractive strategy for improving the bioavailability of poorly absorbed drugs.

This review focuses on the physiological characterization and possible molecular mechanisms of the intestinal brush-border and basolateral membrane transport of various natural compounds (i.e., amino acids, oligopeptides, monosaccharides, inorganic phosphate, monocarboxylic acids, bile acids, and several water-soluble vitamins). We also describe the physiological function of the primary active transporter P-glycoprotein, which is expressed at the brush-border membrane of intestinal epithelium, and acts as a barrier to the intestinal absorption of drugs by producing net basolateral-to-apical flux of xenobiotics. The feasibility of drug absorption, for either parent drugs or appropriately modified drugs, via these transporters is discussed. The transport mechanisms for nutrients and drugs described here are schematically illustrated in Fig. 1.

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SECONDARY ACTIVE TRANSPORT DRIVEN BY Na^+ OR H^+ GRADIENT

Studies with intestinal brush-border membrane vesicles (BBMVs) demonstrate that electrochemical gradients of Na^+ and H^+ play a major role in absorption of glucose, amino acids, bile acids and phosphate (Na^+-gradient dependency) and di/tripeptides, lactic acid, short chain fatty acids and nicotinic acid (H^+-gradient dependency) across the apical membrane of the intestinal epithelial cells (1). An inwardly directed Na^+-gradient can be maintained in living epithelial cells by Na^+, K^+-ATPase present in the basolateral membrane, resulting in an intracellular Na^+ concentration that is lower, by about 10–20 mM, than the extracellular concentration of 100–140 mM (see Fig. 1). Additionally, there is clear evidence for the presence of an H^+-gradient across the intestinal brush-border membrane in the lumen-to-cytoplasm direction. The pH in the close vicinity of the external surface of the brush-border membrane is acidic compared to the pH of the bulk of the luminal fluid. This pH has been determined to be 5.5–6.0 by using microelectrodes in human and in laboratory animals and is known as the "acid microclimate" on the intestinal surface. Since the intracellular pH in the enterocyte is approximately 7.0–7.2, the concentration of H^+ at the luminal side of the brush-border membrane is at least 10 times greater than the cytoplasmic H^+ concentration (2). The Na^+-H^+ exchanger localized in the brush-border membrane is primarily responsible for the intestinal microclimate pH (3). The exchanger catalyzes the entry of Na^+ from the lumen into the enterocytes in exchange for the exit of H^+ from the cell into the lumen.

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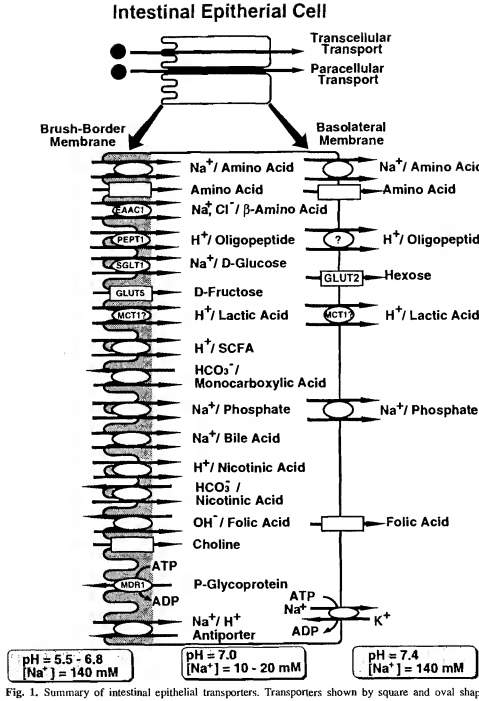

Fig. 1. Summary of intestinal epithelial transporters. Transporters shown by square and oval shapes demonstrate active and facilitated transporters, respectively. The name of cloned transporters were shown within square or oval shapes. In the case of active transporters, the same direction of arrows represent symport of substrate and the driving force. Arrows going in the reverse direction mean the antiport.

TRANSPORT OF AMINO ACIDS AND OLIGOPEPTIDES

Amino Acid Transport

Recently Ganapathy et al. classified amino acid transport systems in the brush-border membrane of small intestine (4). Table I lists their substrate specificity and dependence on ion gradients. However, this classification has not yet been generally accepted. On the other hand, the amino acid transport systems in the intestinal basolateral membrane are classified according to the traditional nomenclature (5) applicable to the plasma membrane of nonpolarized cells (Table II).

System B accepts as substrates nearly all dipolar amino acids that possess the amino group in the α-position. System $B^{0,+}$ is similar to System B, though it accepts not only dipolar amino acids (e.g., leucine), but also basic amino acids (e.g., lysine) as substrates. An inwardly directed Na^+ gradient and an inside-negative membrane potential provide the driving force

Table I. Classification of Amino Acid Transport Systems in the Brush-border Membrane of the Small Intestine (Cited from Ref. 4)

Transport System	Substrates	Dependence on Na^+ Gradient	Involvement of Other Ions
B	Dipolar α-amino acids	Yes	None
$B^{0,+}$	Dipolar α-amino acids Basic amino acids Cystine	Yes	None
$b^{0,+}$	Dipolar α-amino acids Basic amino acids Cystine	No	None
y^+	Basic amino acids	No	None
IMINO	Imino acids	Yes	Cl^-
β	β-Amino acids	Yes	Cl^-
X^-_{AG}	Acidic amino acids	Yes	K^+

for system B and system $B^{0,+}$. The lack of Na^+ dependence is the primary characteristic that distinguishes system $b^{0,+}$ from system $B^{0,+}$. System y^+ transports basic amino acids by an Na^+-independent mechanism and differs from system $b^{0,+}$. The IMINO system accepts exclusively imino acids such as proline, hydroxyproline and pipecolic acid in an Na^+- and Cl^--dependent manner. The β-system accepts nonprotein amino acids, taurine and other β-amino acids and has no affinity for α-amino acids, or acidic/basic amino acids. The IMINO system and the β-system each require both inwardly directed Na^+ and Cl^- gradients as driving forces (6,7). System X_{AG}– accepts acidic amino acids, glutamate and aspartate (4).

Human carcinoma cell line Caco-2 has the Na^+-dependent and -independent amino acid transport systems described above. Caco-2 cells were recently demonstrated to express, at the apical membrane, novel Na^+-independent and H^+-coupled transport systems recognizing β-alanine, L-alanine, proline and α-methylaminoisobutyric acid (8).

The intestinal amino acid transport system, y*, was the first mammalian amino acid transporter to be successfully cloned. It is a protein with 622 amino acid residues and is predicted to have 14 membrane-spanning domains. Injection of cRNA transcribed from the cloned cDNA into *Xenopus laevis* oocytes leads to an increase in the Na^+-independent transport of cationic amino acids, lysine, arginine and ornithine, but does not induce cysteine transport. The Na^+-independent amino acid transport system, $b^{0,+}$, has been cloned from rat and rabbit kidney, and is also expressed in the small intestine. This transport protein referred to as NBAT, exhibits no homology with system y^+. A cDNA for rabbit intestinal Na^+-dependent glutamate transporter, referred to as EAAC1, was isolated and shown to encode a 524 amino acid protein predicted to have ten membrane-spanning domains (9).

Table II. Classification of Amino Acid Transport Systems in the Basolateral Membrane of the Small Intestine (Cited from Ref. 4)

Transport System	Substrates	Dependence on Na^+ Gradient
A	Dipolar α-amino acids Imino acids	Yes
ASC	Three- and four-carbon dipolar amino acids	Yes
asc	Three- and four-carbon dipolar amino acids	No
L	Bulky, hydrophobic, dipolar amino acids	No
y^+	Basic amino acids	No

Amino Acid-mimetic Drug Absorption via Amino Acid Transporters

Gabapentin (structure in Fig. 2), an analogue of GABA with neuroprotective action and antiepileptic properties, is absorbed slowly following oral administration, with a decreased absorption from 74% to 36% as the gabapentin dose is increased from 100 to 1600 mg. Studies with rat intestinal perfusion and everted rat intestinal rings indicated that gabapentin is absorbed from the small intestine, though not efficiently, by the transporter for large neutral amino acids (10).

Several amino acid analogues (Fig. 2) such as α-methyldopa (11,12), L-dopa (13) and baclofen (14) by large neutral amino acid transporter and D-cycloserin (15) by proton-coupled amino acid transporter, respectively, are also absorbed from the small intestine via each amino acid transport system.

Oligopeptide Transport

Although it has long been believed that intestinal peptide transport can be energized by the Na^+ gradient, H^+ gradient dependence has now been widely accepted, and the phenomenon has been confirmed in various animal species including human and with different tissue preparations. The coupling of intestinal peptide transport to H^+ rather than Na^+ is an important distinction between intestinal peptide transport and amino acid transport (16). As clearly shown in Fig. 3, in the human intestinal brush-border membrane vesicles an inwardly directed H^+ gradient markedly stimulates the transport of the dipeptide glycyl-glutamine but does not affect the transport of the amino acid glutamine (17).

An H^+-coupled peptide transporter, PepT1 was cloned in 1994 from rabbit intestine (18) and in 1995 from human intestine (19). The cDNA encodes 707 and 708 amino acid residues for rabbit and human PepT1, respectively, with twelve putative membrane-spanning regions and an unusually large hydrophilic loop having several N-glycosylation sites (Fig. 4). We have also cloned the homologue of rabbit PepT1 gene from a rat intestinal cDNA library. The cDNA sequence of rat PepT1 is composed of 710 amino acids and the predicted amino acid sequence shows 77% and 83% identity with rabbit and human PepT1, respectively (20).

Studies of complementary RNA (cRNA) of rabbit (18) and human intestinal PepT1 (19) in *Xenopus laevis* oocytes revealed that [^{14}C]glycylsarcosine (gly-sar) transport was enhanced in the presence of an inwardly directed H^+-gradient. Measurements of intracellular pH in oocytes impaled with pH microelectrodes revealed that the peptide transport is associated with intracellular acidification. Stoichiometric studies showed that each gly-sar molecule is co-transported with one H^+, giving a 1:1 stoichiometry. PepT1 is predicted to be able to concentrate neutral oligopeptides up to about 300-fold across brush-border membranes at the extracellular pH of 5.5 (18). Studies on rabbit PepT1-mediated transport by two-microelectrode voltage-clamp analysis of rabbit PepT1 cRNA-injected oocytes revealed that small peptides containing either neutral, basic or acidic amino acids are transport substrates and that peptides larger

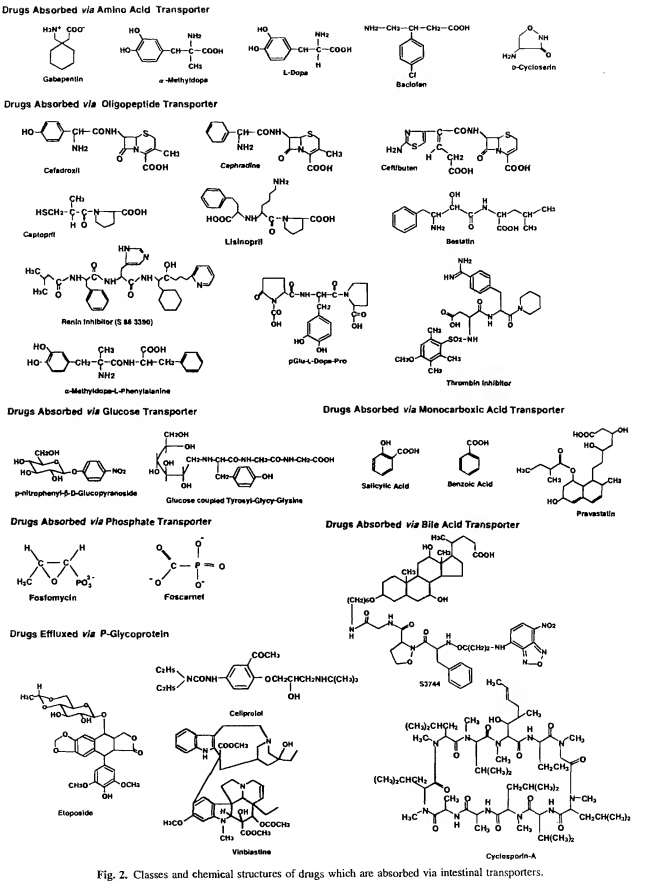

Fig. 2. Classes and chemical structures of drugs which are absorbed via intestinal transporters.

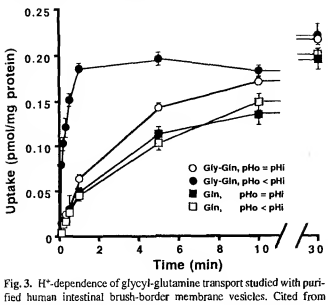

Fig. 3. H^+-dependence of glycyl-glutamine transport studied with purified human intestinal brush-border membrane vesicles. Cited from Ref. 4.

than tetrapeptides are not transported. Similarly, [^{14}C]gly-sar uptake induced with cRNA injection into oocytes of our cloned rat intestinal PepT1 was specifically inhibited by dipeptides and tripeptides, but not by their constituent amino acids or by tetra- or pentapeptides (21).

In contrast to transport across the brush-border membranes, only limited information is available about oligopeptide transport across the basolateral membranes of the intestine. Uptake of [^{14}C]glycyl-L-proline by rabbit proximal intestinal basolateral membrane vesicles oriented inside-out was stimulated in the presence of an inwardly directed pH gradient and followed Michaelis-Menten kinetics with a Km value of 2.0 mM. The uptake was significantly inhibited by 10 mM glycyl-dipeptides and 10 mM cephradine, suggesting the existence of a proton-coupled oligopeptide transporter in the intestinal basolateral membrane similar to that in the brush-border membrane (22). Basolateral membrane of Caco-2 cells possesses an electrogenic H^+-coupled dipeptide transporter, as demonstrated by measuring the transport of [^{14}C]gly-sar and monitoring dipeptide-stimulated H^+-influx across the basolateral membrane (23). The localization of rabbit PepT1 has been confirmed with PepT1 antibody to be limited to the intestinal apical membrane (Tsuji et al., unpublished observation), suggesting the existence of a different H^+/oligopeptide transporter(s) from PepT1 in the intestinal basolateral membrane.

An isoform of PepT1, PepT2, has been recently isolated from human kidney and shown to be ~50% identical and 70% similar to PepT1. Functional expression of the kidney cDNA in HeLa cells resulted in the induction of an H^+-coupled transport specific for di- and tri-peptides and aminocephalosporins. This transporter PepT2 is mostly expressed in the kidney, but not in the small intestine (24).

Absorption of Peptide-mimetic Drugs via Oligopeptide Transporter

Many studies have shown that certain hydrophilic β-lactam antibiotics (structures in Fig. 2) can be transported by oligopeptide transporters in intestinal tissue preparations, isolated intestinal brush-border membrane vesicles, and Caco-2 cells (25). As shown in Fig. 5, a clear overshoot phenomenon was observed for cephradine in rabbit intestinal brush-border membrane vesicles, when an inwardly directed H^+ gradient was imposed (26). Such a stimulated uptake in the presence of an inwardly directed H^+ gradient was reduced significantly when FCCP, a protonophore, was preloaded (Fig. 5, Panel B) or when various dipeptides were added, suggesting that cephradine was taken up by the brush-border membrane H^+/oligopeptide transporter(s). Efflux of cephradine from Caco-2 cells is also via a carrier-mediated process (27), probably via the basolateral H^+/oligopeptide transport system described above.

We have reported the expression of a transporter for both zwitterionic (cefadroxil) and di-anionic (ceftibuten) β-lactam antibiotics in *Xenopus laevis* oocytes injected with mRNA obtained from rat, rabbit and human small intestinal epithelial cells (28,29). The transporter expressed in oocytes was considered to be an H^+/oligopeptide transporter, as judged from the pH-dependence of the transport activity for several β-lactam antibiotics and from the substrate specificity evaluated on the basis of inhibitory and countertransport effects. The transport was stereospecific, i.e., uptake of the *cis*-isomer (ceftibuten) was stimulated in rat intestinal brush-border membrane vesicles in the presence of H^+ gradient, but that of the *trans*-isomer was not in oocytes which expressed an H^+/oligopeptide transporter(s) after injection of rat intestinal mRNA.

Our cloned rat PepT1 exhibited transport activity for β-lactam antibiotics, cephalexin, cephradine, cefadroxil, cefixime, and ceftibuten (*cis*-isomer) but not for cefazolin or the *trans*-isomer of ceftibuten (Fig. 6). The mutual inhibitory effects between dipeptides and β-lactam antibiotics on uptake by rat PepT1 expressed in *Xenopus laevis* oocytes indicate possibly common binding site(s) on the PepT1 (21). Evidence for H^+/cefadroxil transport activity, which was inhibited by cephaloglycine, cefaclor, ampicillin, enalapril, and captopril, but not by cefamandol, cephalothin, benzylpenicillin, or lisinopril at the concentrations of 10 mM, was also observed in oocytes expressing rabbit PepT1 (30). These results show that orally absorbed cephalosporins, aminopenicillins, captopril, and enalapril, but not parenterally used cephalosporins, benzylpenicillin, and lisinopril, are substrates for the H^+/oligopeptide transporter.

Several peptidomimetic drugs (see Fig. 2) other than β-lactam antibiotics, i.e., angiotensin-converting enzyme inhibitors such as captopril, enalapril, lisinopril (30–32), renin inhibitors (33,34), anticancer drugs such as bestatin (35), and peptidomimetic thrombin inhibitors (36), have been proposed to be taken up by the intestinal H^+/oligopeptide transporter.

The intestinal peptide transport system can be employed to improve intestinal absorption of certain drugs by chemically converting them to di- or tripeptide type prodrugs. The very low bioavailability of α-methyldopa, which is taken up by Na^+-coupled neutral amino acid transporter has been improved by the use of dipeptide prodrugs, such as α-methyldopa-L-phenylalanine (Fig. 7), which can be taken up by the intestinal H^+/oligopeptide transporter (31,32,37). To overcome the low bioavailability of oral L-dopa due to decarboxylation in the gut wall, a tripeptide prodrug of L-dopa, p-glu-L-dopa-pro was designed to be absorbed via the intestinal peptide transporter, so as to minimize the decarboxylation in the gut wall, and to be converted to L-dopa by peptidases, with cleavage by

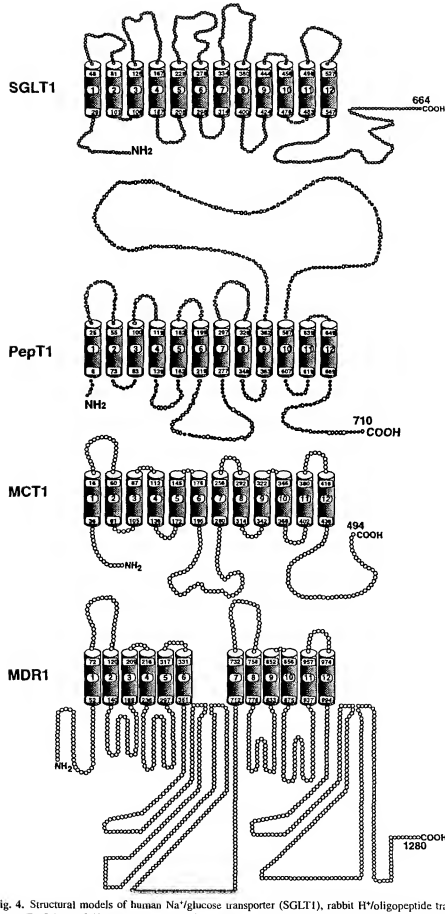

Fig. 4. Structural models of human Na^+/glucose transporter (SGLT1), rabbit H^+/oligopeptide transporter (PepT1), rat H^+/monocarboxylic acid transporter (MCT1) and human MDR1. Cited from Ref. 9, Ref. 65, and Ref 110.

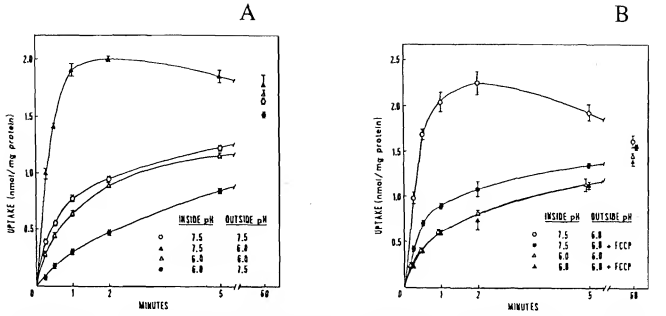

Fig. 5. Effects of outer medium pH (Panel A) and FCCP (Panel B) on cephradine uptake by rabbit intestinal brush-border membrane vesicles. Cited from Ref. 26.

pyroglutamyl aminopeptidase I to L-dopa-pro as the rate-limiting step (38).

Therefore, it is clear that the design of prodrugs suitable for transport by the peptide transporter can be a useful strategy for improving the absorption of small polar drugs which exhibit very poor bioavailability.

CARBOHYDRATE TRANSPORT

Many membrane transport studies on carbohydrates have shown that three mechanisms, active transport, facilitated transport, and passive diffusion/paracellular transport, operate in parallel for the transfer of these hexoses into blood stream. Although several studies have indicated a significant participation of paracellular absorption (39), more studies are needed to clarify the relative importance of saturable and nonsaturable

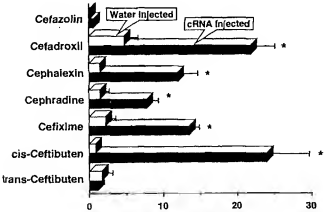

Fig. 6. Uptake of cephalosporins (2 mM) by *Xeopus laevis* oocytes injected with rat intestinal H⁺/oligopeptide transporter, PepT1 cRNA (closed column) or water (open column) at 27°C. Cited from Ref. 21.

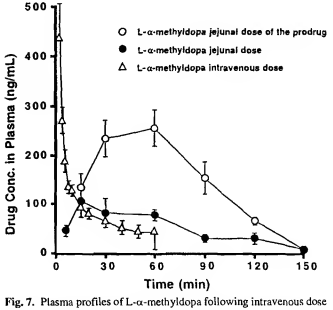

Fig. 7. Plasma profiles of L-α-methyldopa following intravenous dose of L-α-methyldopa and jejunal dose of L-α-methyldopa-phenylalanine and L-α-methyldopa. Cited from Ref. 32.

transport mechanisms in the intestinal absorption of monosaccharides under physiological conditions.

Sodium Ion-Dependent Active Transport of Monosaccharides

Active transport of D-glucose across the intestinal brush-border is energized by the electrochemical gradient of sodium ions. The sodium ion-D-glucose cotransporter has been cloned by the expression cloning technique using a heterologous gene expression system in *Xenopus laevis* oocytes, and it was named SGLT1 (40). SGLT1 is a hydrophobic integral membrane pro-

tein with approximately twelve putative membrane-spanning domains (Fig. 4). Homologous clones were isolated for SGLT1 from rat, pig and human (41,42).

Facilitated Transport of Monosaccharides

D-Fructose is absorbed slowly but significantly from the intestine. A cDNA clone encoding GLUT5, a candidate for the D-fructose transporter, was isolated from a human jejunal cDNA library (43). D-Fructose transport was sodium-independent and not inhibited by D-glucose, D-galactose, sucrose, or α-methylglucopyranoside. GLUT5 cDNA was also cloned from rabbit and rat small intestines, and the expressed proteins showed functional similarities with human GLUT5 (44,45). From these results, the intestinal brush-border membrane transport of hexoses can be ascribed to a sodium-dependent active transporter SGLT1 for D-glucose, D-galactose, and their analogues and a facilitative transporter GLUT5 for D-fructose.

Basolateral Transport of Monosaccharides

Monosaccharides accumulated in enterocytes are transported by a facilitative transporter across the basolateral membranes into blood. From cDNA libraries of rat liver and human liver, cDNA for the glucose transporter GLUT2 was cloned (46,47). The transporter was suggested to exist in the intestine and to have a 2-deoxy-D-glucose transport activity. Immunofluorescence analysis by using GLUT2 antibodies (48) revealed the presence of the protein only in the intestinal basolateral membrane, not in the brush-border membrane. Accordingly, transport of glucose across intestinal basolateral membranes appears to be mediated by the facilitative transporter GLUT2. Intestinal epithelial transport of monosaccharides has been well reviewed (9).

Utilization of Monosaccharide Transporters for Drug Absorption

There have been several attempts to facilitate intestinal absorption and tissue distribution of less permeable compounds by utilizing monosaccharide transport systems, through modification of parent compounds to sugar analogues. Permeation of *p*-nitrophenyl β-D-glucopyranoside (Fig. 2) across rat everted jejunum was comparable with that of D-glucose and was significantly reduced in the presence of phlorizin and by replacement of sodium ions in the incubation medium with potassium ions (49). Interestingly, permeation of *p*-nitrophenyl β-D-galactopyranoside was lower than that of glucose conjugates (50), which is consistent with the affinity of monosaccharides for SGLT1; namely, glucose has a higher affinity than galactose.

A strategy for the enhancement of intestinal absorption by derivatization to monosaccharide analogues was also applied to peptides. Mono- or disaccharide derivatives of tyrosyl-glycyl-glycine appeared on the serosal side and no metabolites of them were detected following the addition of these compounds to the mucosa of intestines. Although the improved intestinal absorption has not yet been definitively ascribed to the intestinal sugar transporters, the coupling of unstable peptides with sugars does improve both hydrolytic stability and membrane permeation (51). A much larger peptide, insulin was also modified with *p*-nitrophenyl-α-D-gluco-pyranoside and *p*-nitrophenyl-α-D-mannopyranoside, where the expected hypoglycemic effects were observed after intra-intestinal administration in rats. In contrast, the *p*-nitrophenyl-α-L-arabino-pyranoside-insulin derivative was not effective. Intestinal absorption of insulin apparently occurred following modification of insulin with sugar, and may reflect both increased resistance to enzymatic hydrolysis and enhanced membrane permeation (52).

MONOCARBOXYLIC ACID TRANSPORT

Passive Diffusion by pH-Partition and pH-Dependent Carrier-Mediated Transport

Apparent increase in the intestinal absorption of weak organic acids with decreased pH has been empirically explained by pH-partition theory, by assuming that the un-ionized form of the acid permeates passively through the intestinal epithelial membranes. However, a significant shift of apparent pKa from the true value to a more alkaline pH, as evaluated from the pH-absorption rate profile, is often observed in the intestinal absorption of weak organic acids, resulting in a greater absorption than expected from the theory (53). Several modifications of the theory have been proposed, including participation of paracellular transport of drugs, the presence of a mucosal unstirred water layer and the importance of an acidic microclimate (54). All of these modifications of pH-partition theory retain the assumption that organic weak acids are absorbed by passive diffusion. Although intestinal absorption by passive diffusion definitely occurs, there are several studies which suggest involvement of carrier-mediated transport across intestinal epithelial cells, mainly brush-border membrane, for several natural and synthetic weak organic acids, as described below.

Transport of Lactic Acid and Short-chain Fatty Acids

Lactic acid (pKa 3.86) largely exists as a dissociated form at the pH of intestinal luminal solution and is expected to be transported across plasma membranes by some specialized mechanism, but not by passive diffusion. A predominant role of proton gradient-dependent transport for lactic acid and its analogues over sodium-dependent transport was reported in intestinal brush-border membrane vesicles of humans (55).

Short-chain fatty acids (SCFA), which are also called volatile fatty acids (including acetic acid, propionic acid and butyric acid), are produced in the gut by microbial digestion of carbohydrates. The intestinal absorption of SCFA is now ascribed to both non-ionic passive diffusion and carrier-mediated transport (56). As a pH-dependent carrier-mediated absorption mechanism for SCFA, a proton-cotransport system was postulated to function in rabbit intestinal brush-border membrane vesicles (57). In that study, initial uptake of [^{3}H]acetic acid (4.5 μM) by the brush-border membrane vesicles increased with decreased pH from 7.5 to 5.0, as shown in Fig. 8. The uptake was completely abolished in the presence of an excess amount of unlabeled acetic acid (500 μM). The initial uptake of [^{3}H]acetic acid by protein-free liposomes made from egg yolk lecithin was also increased at acidic pH. However, the addition of unlabeled acetic acid did not affect the uptake by the liposomes. Accordingly, the decrease in the uptake of [^{3}H]acetic acid by the intestinal brush-border membrane vesicles in the presence of unlabeled acetic acid is considered to be due to a specific carrier-mediated transport mechanism, presumably a proton-cotransport mechanism, but not via passive diffusion.

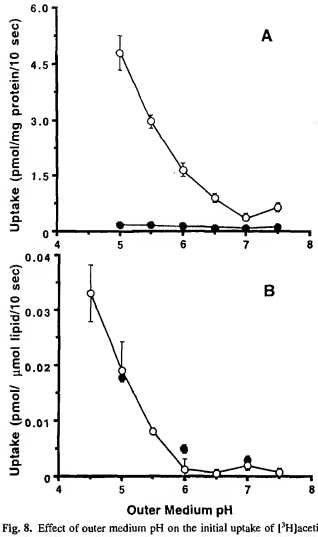

Fig. 8. Effect of outer medium pH on the initial uptake of [^{3}H]acetic acid by rabbit intestinal brush-border membrane vesicles (A) and by egg yolk liposomes (B). The initial uptake rates of [^{3}H]acetic acid were determined at 10 sec at 27°C in the presence (closed circles) or absence (open circles) of an excess amount of unlabeled acetic acid (0.5 mM). The concentrations of [^{3}H]acetic acid used were 4.5 µM for the membrane vesicle study (A) and 8.3 µM for the liposome study (B). In intestinal brush-border membrane vesicles, [^{3}H]acetic acid uptake was significantly reduced in the presence of unlabeled acetic acid, whereas no reduction was observed in the uptake by liposomes. Cited from Ref. 57.

That the SCFA influx is coupled with an efflux of bicarbonate was suggested in rat and human colon by the observation of an enhanced alkalinization in intestinal luminal fluid by SCFA, using an Ussing-type chamber (58). We also observed similar enhancement of luminal alkalinization upon addition of acetic acid to the luminal bathing solution using rabbit small intestinal segment (unpublished observation in our laboratory). Anion exchanger-mediated transport of acetic acid was also demonstrated in the intestinal brush-border and basolateral membrane vesicles from herbivorous teleost (59) and from rabbit (60) and similar transport of propionic acid was found for humans (61). Interestingly, in those studies, such exchange transport of SCFA with bicarbonate was apparently enhanced at acidic extravesicular pH. Anion exchanger-mediated transport of SCFA is likely to be affected by microclimate pH at the intestinal membrane surface in vivo.

Recently, the rabbit erythrocyte lactic acid transporter was purified and a partial N-terminal amino acid sequence was obtained (62). The molecular size of the transporter is 40–50 kilodaltons and the N-terminal sequence was PPAVGGPV-GYTPPDGG. Interestingly, this sequence is identical to the predicted N-terminal sequence of the protein encoded by cDNA for the proton-coupled monocarboxylic acid transporter, MCT1, cloned from Chinese hamster ovary (CHO) cells (63). By northern blot hybridization using CHO MCT1 as the probe, we identified MCT1 in rat and rabbit intestines and in Caco-2 cells (64). The size of mRNA in rats and rabbits hybridized with CHO-MCT1 was about 3.4 kilobases, which is consistent with that found in CHO cells. We screened a rat intestinal cDNA library by using CHO-MCT1 as the probe and obtained the rat MCT1 cDNA (3.4–3.6 kilobases) (Fig. 4). The cDNA was inserted into pBluescript SK(−) and the cRNA was synthesized. When cRNA encoding rat MCT1 was injected into *Xenopus laevis* oocytes, stereospecific and pH-dependent transport activities for lactic acid (65), as well as for SCFA such as acetic acid and pyruvic acid were observed (Tsuji et al., unpublished observation). Thus, it is highly likely that MCT1 cloned from the rat intestine plays a role in the transport of lactic acid, SCFA, and other monocarboxylic acids in the small intestine.

Transport of Monocarboxylic Acid-type Drugs

Many synthetic monocarboxylic acid compounds have been thought to be absorbed mainly by passive diffusion according to the pH-partition theory. In this section, several lines of evidence are presented to suggest participation of carrier-mediated transport mechanisms for monocarboxylic acid drugs.

Intestinal absorption of salicylic acid has been studied in detail, which suggest both specific carrier-mediated transport and passive diffusion participate in salicylate transport (53,66). Transcellular transport of salicylic acid and benzoic acid across Caco-2 cells showed several characteristics of carrier-mediated transport mechanisms (67,68). In salicylic acid transport, saturability and *cis*-inhibitory effects by structural analogues were observed. Interestingly, a fairly good correlation was observed between intestinal absorption rate constants obtained by the *in situ* rat intestinal loop method and apparent affinity for the putative transporter evaluated from the inhibitory potencies of several salicylic acid analogues (67). All of these observations suggest that a carrier-mediated transport mechanism is important for the transport of benzoic acid and salicylic acid in Caco-2, and a similar mechanism may function in intestinal absorption.

Pravastatin, a water-soluble 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is classified as a monocarboxylic acid and has a pKa of 4.7. Its bioavailability after oral administration is fairly high, and other monocarboxylic acid-type HMG-CoA reductase inhibitors also show good bioavailability, comparable with those of lipid-soluble prodrug (lactone) analogues. Uptake of pravastatin by brush-border membrane vesicles was increased at acidic pH and showed an overshoot in the presence of an inwardly-directed proton gradient. The uptake rate estimated for 10 sec was saturable with a Km of 15 mM at an extravesicular pH of 5.5. Pravastatin uptake was reduced in the presence of several monocarboxylic

acids such as mevalonic acid, benzoic acid, and monocarboxylic acid forms of structural analogues, lovastatin acid and simvastatin acid, whereas di- and tricarboxylic acids or acidic amino acid were not inhibitory. Furthermore, mutual inhibitory effects were observed between pravastatin and acetic acid (69). Based on these results, a proton-coupled, monocarboxylic acid-specific transport mechanism for pravastatin is highly likely to be present in the intestine.

PHOSPHATE TRANSPORT

The carrier-mediated transport of phosphate in the small intestine was clearly shown in isolated brush-border membrane vesicles prepared from rat (70) and human intestine (71). Uptake of phosphate by the rat membrane vesicles was stimulated in the presence of an inwardly directed sodium ion gradient and was also affected by pH, with an increased activity at an acidic extravesicular pH of 6 compared with neutral pH, 7.4. There have been a few studies on intestinal basolateral membrane transport of phosphate, and a sodium-dependent transport mechanism has been suggested for rat and human intestines (71). However, the precise mechanisms involved remain unclear.

The intestinal phosphate transporters have not been cloned yet. Interestingly, no genes in mammalian intestines homologous to the renal phosphate transporter genes have been found. Accordingly, in contrast to other nutrient transporters, intestinal and renal brush-border membranes may contain structurally different phosphate transporters.

Utilization of Phosphate Transporter for Drug Absorption

The antiviral drug, foscarnet (phosphonoformic acid, structure in Fig. 2), which is water-soluble with pKa values of 0.49 and 7.27 for phosphate hydroxy groups and 3.41 for the carboxyl group, shows a very high absolute bioavailability of about 95% in rabbits, while approximately 30% absorption in mice and rats and 12 to 22% in humans have been reported (72). Such a high intestinal absorption, in spite of its hydrophilic nature, in rabbits and the highly variable availability among animal species may be ascribed to the involvement of carrier-mediated transport mechanisms. The uptake of radio-labeled foscarnet by rat intestinal brush-border membrane vesicles was sodium ion gradient-dependent, showing an overshoot phenomenon, and was activated at an acidic extravesicular pH in comparison with neutral pH (72). Furthermore, the initial rate of uptake was inhibited by unlabeled foscarnet, phosphate, and arsenate. Such a carrier-mediated transport was also observed in studies of rat intestine mounted in an Ussing-type chamber (73). All of these observations indicate that foscarnet may be absorbed in the small intestine via a carrier-mediated mechanism which is common to phosphate.

Fosfomycin, (−)-(1R,2R)-(1,2-epoxypropyl)phosphonic acid (structure in Fig. 2), is a water-soluble antibiotic, which is administered orally as well as parenterally, though its bioavailability is not high. Studies with rat, rabbit and human intestinal brush-border membrane vesicles showed that this phosphate-mimetic antibiotic is taken up by the Na^+-phosphate cotransporter (74,75). An *in situ* intestinal perfusion study suggested that the carrier-mediated absorption via the phosphate transporter is more important at concentrations of less than 1 mM fosfomycin, considering from Km value for the carrier-mediated transport of 1.13 mM (76).

Considering the structures of fosfomycin, foscarnet, and its analogues (e.g., phosphonoacetic acid and phosphonopropionic acid), relatively small molecules containing a phosphate moiety may be utilized as substrates for the intestinal sodium-dependent phosphate transporter, resulting in enhanced intestinal absorption.

BILE ACID TRANSPORT

Bile Acid Transport Mechanism in Intestinal Brush-Border Membrane

Bile acids are acidic sterols synthesized from cholesterol in the liver. Following synthesis, bile acids are secreted into bile, enter the lumen of the small intestine and are reabsorbed to the extent of more than 95% in the small intestine, predominantly by an Na^+ gradient-driven transporter located at the brush-border membrane of the ileum (77). Recently, cDNA of the bile acid-Na^+ acid cotransporter (IBAT), encoding a 348-amino acid protein with seven putative transmembrane domains and three possible N-linked glycosylation sites, was cloned (78). The amino acid sequence was 36% identical and 63% similar to that of the rat liver bile acid-Na^+ transporter (LBAT).

Utilization of Bile Acid Transporters for Drug Absorption

A series of small, linear, model peptides up to a chain length of 10 amino acids were covalently coupled to the 3-position of a modified bile acid yielding peptidyl-3β-(ω-aminoalkoxy)-7α,12α-dihydroxy-5β-cholan-24-oic acid (structure in Fig. 2). These compounds and a bile acid conjugate (S3744) were able to interact with the ileal bile acid-Na^+ cotransport system, as was shown by their concentration-dependent inhibitory effects on Na^+-dependent [^{3}H]taurocholate uptake by brush-border membrane vesicles from rabbit ileum (79).

DRUG ABSORPTION VIA WATER-SOLUBLE VITAMIN TRANSPORT SYSTEMS

Drug Absorption via Nicotinic Acid Transport System

Nicotinic acid has a single carboxyl group with a pKa of 4.9. A rat intestinal brush-border membrane vesicle study revealed a sodium-independent and proton-gradient-dependent transport system for nicotinic acid (80), although previous studies using everted sacs or perfusion of rat small intestine showed passive diffusion or Na^+-dependent transport. Nicotinic acid uptake was inhibited by several acidic compounds including acetic acid, valproic acid, benzoic acid and salicylic acid. These effects were considered to be specific, because the transport of acetic acid and benzoic acid by brush-border membrane vesicles or Caco-2 cells, respectively, was also inhibited by nicotinic acid (67,68). Nicotinic acid transport in intestinal brush-border membrane vesicles was also stimulated in the presence of an outwardly-directed bicarbonate gradient at acidic pH (81). Several lines of evidence support the hypothesis that various monocarboxylic acid-like drugs, such as valproic acid, salicylic acid, and penicillins, are absorbed via H^+-nicotinic acid cotransporter

and/or HCO_3^-/nicotinic acid exchanger as well as lactic acid and/or SCFA transporters, as described in the previous section.

Transport of Folic Acid and Its Analogues

Folic acid is likely to be absorbed mainly via a pH-dependent carrier-mediated transport mechanism (82). In rat, rabbit, and human intestinal brush-border membrane vesicles, an inwardly-directed proton-gradient induced overshoot uptake of folic acid, and the uptake was saturable and inhibited by an anion exchange inhibitor, DIDS. These results were explained in terms of a folic acid-hydroxyl exchange or proton-cotransport mechanism (83). Basolateral membrane transport of folic acid was reported to be saturable, electroneutral, sodium-independent and sensitive to DIDS (84).

Methotrexate is an analogue of folic acid and its intestinal absorption mechanism is similar to that of folic acid. However, it has also been suggested that there are multiple pathways for methotrexate, one being common with folic acid and the other specific to methotrexate due to the presence of folic acid-insensitive flux as a major component of the total flux of methotrexate, though the precise mechanism of the transport has not been clarified yet (85). Although potocytosis has also been suggested in an internalization of folic acid via folic acid receptor (86), it is not clear whether the mechanism functions in intestinal epithelial cells for the absorption of methotrexate or not.

Drug Absorption via Choline Transport System

Recent studies using isolated brush-border membrane vesicles confirmed a facilitated transport of choline in rats (87). Uptake of choline by membrane vesicles was not sensitive to an inwardly directed sodium or proton gradient, membrane potential or outwardly directed proton gradient, but was saturable with a Km of 159 μM. Tetraethylammonium, acetylcholine, and N-methylnicotinamide, which are comparatively water-soluble, small organic cations, most likely share a common transporter with choline, whereas hydrophobic hexyltrimethylammonium and octyltrimethylammonium probably do not (87). Furthermore, the presence of a carboxyl group in choline analogues, including betaine, carnitine, sarcosine, and N,N'-dimethylglycine, decreases affinity for the choline transporter (88).

DRUG ABSORPTION LIMITED BY P-GLYCOPROTEIN-MEDIATED SECRETORY DRUG TRANSPORT

P-Glycoprotein (P-gp, the putative structure of human *MDR1* gene is shown in Fig. 4) is a transmembrane protein of 170 kDa associated with a phenotype of multidrug resistance (MDR) of tumor cells to certain anticancer agents through pumping the agents out of the cells, thereby reducing the intracellular accumulation of the drugs. Functionally, P-gp is characterized by a surprisingly broad substrate specificity, including anticancer drugs, calcium channel blockers, immunosuppressive agents and others, and is classified as an ATP-dependent primary active transporter belonging to the ABC (ATP binding cassette) transporter superfamily. Furthermore, P-gp has been shown to be present and to function as a transporter in plasma membrane of many normal tissues (89,90). The functional significance of P-gp as the drug efflux pump in some normal tissues was clearly demonstrated by generating a mouse strain in which the mdr1a gene encoding P-gp was disrupted. In the mouse lacking the mdr1a gene product, distribution of an anticancer drug, vinblastine, and an anthelmintic agent, ivermectin, was enhanced in many tissues especially in the brain (91), and this result supports our previous conclusion that P-gp has a function in maintaining the blood-brain barrier (92–95). Intestinal tissue distributions of vinblastine and ivermectin were also increased in the mutant mouse (91). This increase is consistent with the previous findings that P-gp is localized on the luminal membrane of intestinal epithelial cells and transports anticancer drugs such as anthracyclins (96–98) and etoposide (99), antibiotic agents such as pristinamycin (100), peptides such as cyclosporin A (101) and model peptides (102), β-blockers such as celiprolol (103) and other organic cations (104,105). These results have been obtained by measurements of the polarized transport from the basolateral to apical side across isolated intestinal tissues or cultured Caco-2 cells and the effect of classical P-gp inhibitors such as verapamil and functionally blocking-type anti-P-gp antibody on the transepithelial transport (106).

From the above data, it is clear that P-gp functions to reduce apparent intestinal epithelial permeability from lumen to blood for various lipophilic or cytotoxic drugs. Figure 9 shows a summary of the relationship between absorption clearance evaluated by the rat intestinal perfusion or loop method and lipid solubility of the drugs (107–109). Here, closed squares represent the absorption clearance obtained in the case of simultaneous intravenous administration of cyclosporin A to block the function of P-gp. The compounds examined include β-blockers (atenolol, acebutolol, celiprolol and nadolol), anticancer drugs (doxorubicin and vinblastine) and other P-gp substrates (cyclosporin A, digoxin and verapamil). The solid line represents a visually fitted correlation curve for the drugs shown by circles. Intestinal absorption of drugs involved in the multidrug-resistance phenotype tends to be increased to some extent by cyclosporin A administration, although the increased rate constants still seem to be lower than those expected from the apparent correlation (solid line) (unpublished observations in our laboratory). Thus, P-gp primarily decrease net intestinal absorption of some drugs, although other secretory mechanisms may also contribute to the reduction of apparent intestinal permeability.

CONCLUSION

This review focuses on the experimental basis for the modern approach to carrier-mediated intestinal absorption/secretion of drugs, rather than the classical approach of passive absorption by intestinal tract by increasing the lipophilicity of drugs. In recent years, transport proteins for nutrients have been established to play an important role in regulating the intestinal absorption of xenobiotics. An understanding of the functional characteristics of such transporters should provide information on how transporters contribute to the increased bioavailability of xenobiotic compounds, facilitating the design of new orally effective drugs.

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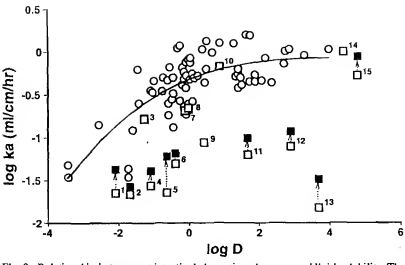

Fig. 9. Relationship between rat intestinal absorption clearance and lipid solubility. The results shown with the squares represent the relationship between intestinal absorption clearance (ka) observed from the in situ jejunum loop in the presence (■) and absence (□) of cyclosporin A in rats and octanol-buffer (pH 7.0) partition coefficients (log D). determined in this study. The results shown with circles were obtained from Refs. 106, 107, and 108. Other data points were determined by our laboratory which include 1, atenolol; 2, nadolol; 3, acetamide; 4, celiprolol; 5, acebutolol; 6, doxorubicin; 7, timolol; 8, sulfathiazole; 9, quinidine; 10, sulfamethoxazole; 11, digoxin; 12, cyclosporin A; 13, vinblastine; 14, β-estradiol; 15, verapamil.

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